DOCTORAL THESIS

Non-antimicrobial strategies for the prevention and treatment of infections by multidrug-resistant Gram-negative bacilli.

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El Dr. Jerónimo Pachón Díaz, Catedrático de Medicina de la Universidad de Sevilla y Jefe de Servicio de Enfermedades Infecciosas del Hospital Universitario Virgen del Rocío, como Director y Tutor de la Tesis Doctoral, y el Dr. Younes Smani, investigador contratado por el programa "Miguel Servet" del Instituto de Salud Carlos III y miembro de la Unidad Clínica de Enfermedades Infecciosas, Microbiología y Medicina Preventiva del Hospital Universitario Virgen del Rocío, como Director de la Tesis Doctoral,

CERTIFICAN:

Que la Tesis para optar al grado de Doctor por la Universidad de Sevilla que lleva por título "***Non-antimicrobial strategies for the prevention and treatment of infections by multidrug-resistant Gram-negative bacilli***" ha sido realizado por la Licenciada Doña Raquel Parra Millán bajo nuestra supervisión, considerando que reúne los requisitos necesarios para su presentación.

Para que conste a los efectos oportunos, expiden la presente certificación en Sevilla, a 13 de marzo de 2017.

Jerónimo Pachón Díaz
Director y Tutor

Younes Smani
Director

“Todo viaje tiene un guía. Y en el de la consecución del éxito,

no se camina sin la constante ayuda del esfuerzo”.

R. Ayerbe

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Congress communications

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Abbreviations

2-AI: 2-aminoimidazole

AHL: Acyl-homoserine lactone

AI-2: Autoinducer 2

AIPs: Auto-inducing oligo-peptides

AMEs: Aminoglycoside-modifying enzymes

ApoE: Apolipoprotein E

BAM: β-barrel assembly machinery

CDC: Centers for Disease Control and Prevention

c-di-GMP: Cyclic diguanylic acid

ChoP: Phosphorylcholine

CRE: Carbapenem-resistant *Enterobacteriaceae*

CSA: Cationic steroid antimicrobial

cup: Chaperone-usher pathway

DDD: Defined daily doses

DPhPC: Diphytanoylphosphocholine

DNA: Deoxyribonucleic acid

EARS-Net: European Antimicrobial Resistance Surveillance Network

ECDC: European Centre for Disease Prevention and Control

ELISA: Enzyme-linked immunosorbent assay

EPB: ESBLs producing *Enterobacteriaceae*

EPINE: Spanish Nosocomial Infections Prevalence Study

ESBLs: Extended-spectrum beta-lactamases

EU/EEA: European Union/European Economic Area

FDA: Food and Drug Administration

G-CSF: Granulocyte colony stimulating factor

GNB: Gram-negative bacilli

HAIs: Healthcare-associated infections

HAP: Hospital-acquired pneumonia

hBD: Human beta-defensins

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

i.p.: Intraperitoneal

ICUs: Intensive care units

IFN-γ: Interferon gamma

Abbreviations

IL-1β, IL-4, IL-6, IL-10: Interleukin-1β, -4, -6, -10

KCl: Potassium chloride

LPC: Lysophosphatidylcholine

LPS: Lipopolysaccharide

MBL: Metallo-β-lactamases

MFS: Major facilitator superfamily

MIC: Minimal inhibitory concentration

MDR: Multidrug-resistant

MLD: Minimal lethal dose

OG: Octyl glucopyranoside

OMPs: Outer membrane proteins

OMVs: Outer membrane vesicles

PAFR: Platelet-activating factor receptor

PBPs: Penicillin binding proteins

PDR: Pandrug–resistant

PRIOAM: Institutional Programme for the Optimization of Antimicrobial Treatment

QS: Quorum sensing

RNA: Ribonucleic acid

SEIMC: Spanish Society of Infectious Diseases and Clinical Microbiology

TLR4: Toll-like receptor 4

TNF-α: Tumor necrosis factor alpha

VAP: Ventilator-associated pneumonia

WHO: World Health Organization

XDR: Extensive drug-resistant

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ABSTRACT

Abstract

Due to the increased of antimicrobial resistance rates and the difficulty of having effective treatment for infections caused by Gram-negative bacilli (GNB) such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Escherichia coli*, it is necessary to develop non-antimicrobial therapeutic alternatives, which can be used together with the scarce and non-optimal antimicrobial available. In this doctoral thesis, and in order to stop the evolution of the bacterial infection, we aimed to evaluate two therapeutic alternatives: i) blocking the virulence factors of *A. baumannii*, *P. aeruginosa* and *E. coli* and ii) modulating the host immune system.

Regarding the first therapeutic approach, we performed the design and evaluation of outer membrane protein A (OmpA) inhibitors on the interaction of *A. baumannii*, *P. aeruginosa* and *E. coli* with the host to block the mechanisms by which this protein produces the infection. Thus, we studied *in vitro* the effect of the lead peptide AOA-2 on the interaction between *A. baumannii*, *P. aeruginosa* and *E. coli* and human lung epithelial cells (A549) by adherence, immunofluorescence, fibronectin binding, and cell viability assays, and also we tested the effect of AOA-2 on biofilm formation by GNB. *In vivo*, the therapeutic efficacy of AOA-2 against *A. baumannii*, *P. aeruginosa* and *E. coli* was evaluated. Moreover, the synergy between AOA-2 and colistin by microdilution assay and time-kill curves was studied, and the outer membrane proteins profile in the presence or absence of the AOA-2 was analyzed. Consequently, we constructed an *A. baumannii knockout* deficient in the *omp25* gene and its complemented strain to perform the same experiments. Finally, we evaluated *in vivo* the therapeutic efficacy of AOA-2 in combination with sub-optimal doses of colistin in peritoneal sepsis caused by *A. baumannii*.

We found that AOA-2 prevented the adhesion of *A. baumannii*, *P. aeruginosa* and *E. coli* to A549 cells protecting them from death, and also reduced biofilm formation by these pathogens. In addition, in the murine peritoneal sepsis model caused by *A. baumannii*, *P. aeruginosa* and *E. coli*, AOA-2 treatment significantly reduced bacteremia, bacterial load in spleen and lung, and mice mortality rates. Regarding the combination of AOA-2 with colistin, the presence of AOA-2 increases the susceptibility of colistin MICs for colistin-susceptible and colistin-resistant *A. baumannii* strains. Time-kill curves showed a synergistic activity between AOA-2 and colistin, and the protein profile exhibited an overexpression of the Omp25 protein in the strains treated with AOA-2. *In vivo*, AOA-2 in combination with colistin reduced bacterial spleen and

lung loads, bacteremia and mortality rates, in comparison with the monotherapy with colistin.

Regarding the second therapeutic approach, we evaluated the effect of lysophosphatidylcholine (LPC), a chemoattractant immunomodulatory factor which stimulates cells from the immune system, as an adjuvant treatment with antimicrobials to treat infections caused by susceptible and MDR strains of *A. baumannii* and *P. aeruginosa* in experimental murine models of peritoneal sepsis and pneumonia. Pharmacokinetics and pharmacodynamics parameters of colistin, tigecycline, imipenem and ceftazidime, and minimal lethal dose (MLD) of each strain were determined. In murine experimental models of peritoneal sepsis and pneumonia, mice were pretreated with LPC, infected with MLD of the correspondence strain, and treated or not with antimicrobials.

We found that LPC in combination with colistin, tigecycline or imipenem shows beneficial effects in infections caused by susceptible and MDR *A. baumannii*. Furthermore, the combination of LPC with ceftazidime or imipenem improves the prognosis of infections caused by MDR *P. aeruginosa*.

The data of this doctoral thesis indicate that both the blocking of bacterial virulence factors and the stimulation of the immune system with AOA-2 and LPC, respectively, alone or in combination with antimicrobials, could protect against infections by GNB.

I. INTRODUCTION

Introduction

1. Current situation of antimicrobial resistance and antimicrobial drugs

Antimicrobial resistance in bacterial pathogens is a worldwide challenge leading high morbidity and mortality in clinical settings (1). The last report by the World Health Organization (WHO) in 2014 warned about this circumstance, and also remarked that there are situations which are increasingly arising where bacteria that are resistant to most, or even all, available antimicrobial drugs are causing serious infections that were readily treatable until recently (2). Among them, we find community-acquired pneumonia, cystitis, common infections in neonatal and urinary-tract infections, the prevention of postoperative surgical site infections and also patients receiving cancer treatment, organ transplant and other advanced therapies that are vulnerable to infection (2).

As reported by the European Centre for Disease Prevention and Control (ECDC), the percentages of microorganisms exhibiting antimicrobial resistance, especially resistance to multiple antibiotics, continued to increase in Europe. Data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) shows large variations in percentages of antimicrobial resistance in Europe depending on microorganism, antimicrobial agent and geographical region. These geographical differences may reflect differences in antimicrobial use and infection control practices in the reporting countries (3).

The situation for GNB is especially worrying with high and, in many cases, increasing resistance percentages reported from many parts of the world. In fact, options for treatment of patients who are infected with such MDR GNB are limited to only a few last-line antibiotics, such as carbapenems (**Figure 1**). However, carbapenem-resistance is an ongoing public-health problem of global dimensions. This type of antimicrobial resistance is spreading rapidly causing serious outbreaks and dramatically limiting treatment options (4).

The use of antimicrobial drugs has become widespread over several decades, and has been extensively misused in both humans and food-producing animals in ways that favor the selection and spread of resistant bacteria (2). In 2013, consumption of antimicrobials for systemic use in the community ranged from 10.8 to 32.0 defined daily doses (DDD) per 1000 inhabitants and per day, depending on the country. In

Spain in 2013, the DDD per 1000 inhabitants and per day was 22.4, while in the EU/EEA population weighted mean was 3.30 DDD per 1000 inhabitants and per day (3).

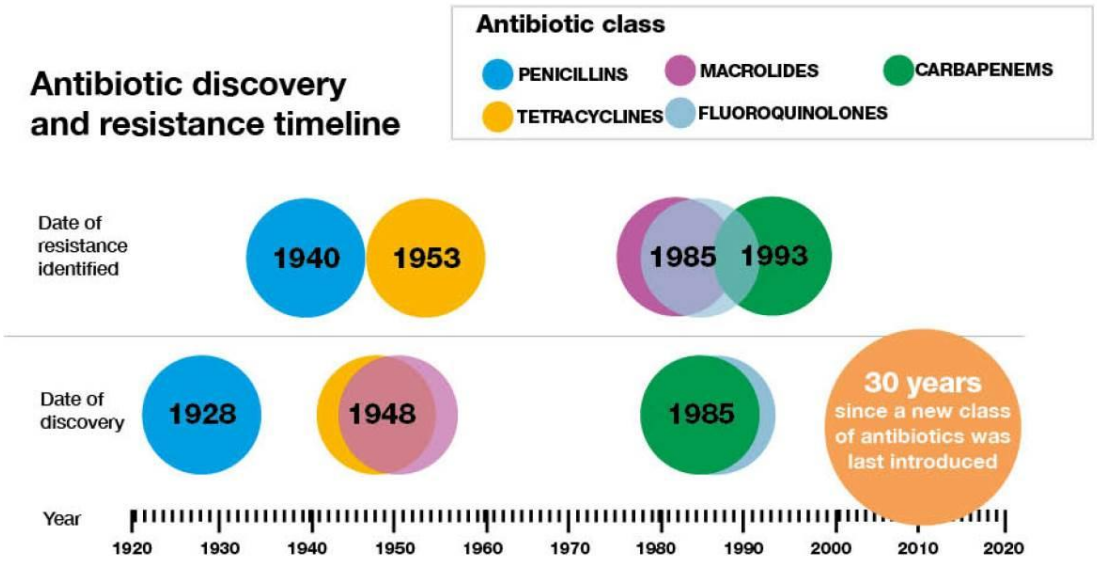

Figure 1. Antibiotic discovery and resistance timeline. Figure taken from Health matters: antimicrobial resistance – GOV.UK.

As in previous years, penicillins were the most frequently prescribed antimicrobial drugs in all countries, whereas the proportion of consumption of other antimicrobial classes varied widely among countries, e.g. cephalosporins and other beta-lactams; macrolides, lincosamides and streptogramins, and quinolones. Countries reporting a high consumption generally have a higher level of antimicrobial resistance than countries reporting a low consumption. Increasingly, EU/EEA countries are implementing actions to control antimicrobial resistance in the community through rational use of antimicrobials, including awareness campaigns on the prudent use of antibiotics. Reliable and comparable antimicrobial consumption data are essential in the evaluation of the effect of such national campaigns. There is a need to improve surveillance of antimicrobial consumption at the level of each individual hospital in EU/EEA countries (3). In fact, in 2011 in Andalusia (Spain), the Institutional Programme for the Optimization of Antimicrobial Treatment (PRIOAM) was launched. This programme is coordinated by a multidisciplinary team chosen by the Committee

on Infections and Antimicrobials and its aim is to educate, train and know the basis for the proper use of antimicrobials in order to reduce mortality and morbidity in patients with infections and to delay the development of resistance (5).

Another debatable issue is the standardization of definitions for acquired resistance. The ECDC and the Centers for Disease Control and Prevention (CDC) created a standardized international terminology to describe acquired resistance profiles. The definition of multidrug-resistance (MDR) is a microorganism that is non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories; extensive drug-resistance (XDR) is considered as non-susceptible to ≥ 1 agent in all but ≤ 2 categories, and pandrug-resistance (PDR) is defined as bacteria resistant to all the antibiotics in all the antimicrobial categories (6, 7).

The high levels and increasing trends of antimicrobial resistance in GNB in Europe highlighted by EARS-Net surveillance results to illustrate the continuous loss of effective antimicrobial therapy especially in healthcare-associated infections (HAIs), and emphasize the need for comprehensive response strategies targeting all health sectors (3).

It is necessary to develop new compounds with activity against MDR GNB, which, together with other measures, would contribute to controlling the current serious situation (8).

2. Healthcare-associated infections (HAIs)

HAIs or nosocomial infections are those infections that appear during hospitalization, manifested after 72 h or more of the admission of the patient in the hospital, and that were neither present nor in period of incubation before the admission of the patient. In intensive care units (ICUs), these infections are sometimes the reason for admission in them, and others are the consequence of staying there (9).

HAIs are a leading cause of morbidity and mortality among hospitalized patients (10). Approximately 4,100,000 patients are estimated to acquire a HAI in the EU each year, and the number of deaths occurring as a direct consequence of these infections is estimated to be at least 37,000 and these infections are thought to contribute to an

additional 110,000 deaths each year (3). They constitute one of the most important problems that may occur in services that take care of critically ill patients, leading to an increased hospital length of stay, higher mortality and a higher health costs (11-15). Due to the situation of the critical ill patient, often immunosuppressed, and high antibiotic selective pressures, the ICU is an important environment for the emergence of antimicrobial drug resistance and the spread of drug-resistant microorganisms (16). Moreover, up to 16 % of HAIs are caused by MDR microorganisms (10), where GNB infections are predominant (55-65 %) with respect to other microorganisms (7).

Bloodstream infections and low respiratory tract infections, such as hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP), cause considerable morbidity and mortality (17-19); otherwise, urinary tract infections are the most common.

The infection of the bloodstream remains a life-threatening situation and is mainly associated with the presence of a central vascular catheter and with a GNB infection in other areas of the body, such as lung, genitourinary tract, or abdomen (17). In fact, in a Spanish clinical study, the attributable mortality for primary and catheter-related bloodstream infections was 9.4 % (20). Almost any GNB can cause bloodstream infection; however, the most common microorganisms include Enterobacteriaceae, *Enterobacter* species, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (17, 21).

HAP is the most common life-threatening HAIs, and the majority of cases are associated with mechanical ventilation. From 10 to 20 % of patients who are on ventilators for longer than 48 hours get VAP (22), and the mortality rates for VAP are estimated to be 30 % to 70 % (10). GNB predominate in HAP, particularly *P. aeruginosa*, *A. baumannii* and the Enterobacteriaceae (23).

In the hospital-acquired urinary infections, GNB are the predominant. Almost all of them are associated with urethral catheterization (24). It has been described that urinary-tract infection account for 20 to 50 % of all HAIs occurring in the ICU (25).

Therefore, it is necessary to establish a system that allows continuous monitoring of the epidemiology, the risk factors for its development and the impact that these infections have in critically ill patients, in order to be able to establish measures for prevention and

control to try to reduce the incidence of nosocomial infections and consequently the significant impact they have in the patient (26).

A considerable attempt that has been suggested is to prevent HAIs through methods designed to reduce the transmission of nosocomial pathogens, such as hand hygiene and isolation of patients with MDR GNB among others (10, 27-29). Furthermore, the Spanish Society of Infectious Diseases and Clinic Microbiology (SEIMC), which has a multidisciplinary approach, approved the creation of the Study Group of Infections in the Critical Patient, whose immediate objectives were to promote research and knowledge, to develop consensus documents with other groups or scientific societies and encourage the teaching and training in specific areas, in which not only would participate intensivists, but also microbiologists, infectious diseases specialists, pharmacists, pharmacologists and all the professionals directly or indirectly related to critical infectious diseases (26).

As mentioned above, most of the HAIs are caused by GNB, predominantly by *A. baumannii*, *P. aeruginosa* and Enterobacteriaceae.

3. Gram-negative bacilli (GNB): epidemiology, antimicrobial resistance and virulence

The burden of antimicrobial resistance in GNB is a daily challenge to face for ICUs staff. Indeed, these pathogens are the most frequent which cause VAP (30). They also cause commonly catheter-related bloodstream infections, and other ICU-acquired sepsis such as surgical site or urinary tract infection (7, 31). The major concerns with antimicrobial resistance and pathogenesis are related to *A. baumannii*, *P. aeruginosa* and Enterobacteriaceae (32, 33).

3.1. *Acinetobacter baumannii*

The genus *Acinetobacter* comprises Gram-negative, strictly aerobic, nonfermenting, catalase-positive, oxidase-negative bacteria with guanine-cytosine content in the DNA of 39 to 47 % (34). Another important feature of this species is its ability to colonize

any type of surface (35). *Acinetobacter* spp. can be found in soil, water, animals and humans (36). This pathogen has been isolated in fresh water ecosystems, air samples from hospitals (37) as well as in kinder gardens and high schools (38). It is known that between the *Acinetobacter* species, *A. baumannii* present the most resistant profile patterns, in fact, is the most common species causing nosocomial outbreaks in hospitals (34). Moreover, *A. baumannii* has the greatest ability to survive desiccation (39).

3.1.1. Clinical and epidemiological importance

The *Acinetobacter* species are mainly opportunistic pathogens. In the ECDC point prevalence survey of HAIs in European acute care hospitals 2011-2012, *Acinetobacter* spp. were the 11th most frequently reported (3.6 %) microorganism in microbiologically documented HAIs (3). More than half of *Acinetobacter* spp. isolates reported by some European countries were resistant to all antimicrobial groups under surveillance (carbapenems, fluoroquinolones and aminoglycosides). Treatment alternatives for patients infected with *A. baumannii* showing combined resistance to carbapenems and other key antimicrobials are confined to combination therapy, and to older antimicrobials such as polymyxins (3).

A. baumannii has high clinical relevance due to the severe infections that it causes, such as VAP (40), bacteremia, skin and soft tissue infections, surgical site infections, urinary tract infections and sepsis (34, 41), and less frequently, meningitis and endocarditis (42, 43). Infections by *A. baumannii* are becoming increasingly frequent. Crude mortality rates of 30-75 % have been reported for nosocomial pneumonia caused by *A. baumannii*, and the mortality attributable to *A. baumannii* infection was found to range from 7.8-43 % (44).

3.1.2. Resistance mechanisms

The resistance of *A. baumannii* to antimicrobial agents has been highly increased in the last decades, which supposes an important problem for the health system reducing or preventing the effect of antimicrobials to treat infected patients. It has become resistant to all the antimicrobial agents used to treat infections by this pathogen due to its ability

to acquire and keep different mechanisms of resistance. The multidrug resistance in *A. baumannii* is a result of the combination of genomic plasticity high level, mutation of endogenous genes, and acquisition of foreign genetic material.

The principal antibiotic resistance mechanisms in *A. baumannii* are β-lactamases hydrolysis, penicillin-binding proteins modification, porins expression loss, efflux pump overexpression, aminoglycoside modifying enzymes activity, mutations in genes codifying DNA gyrase and topoisomerase IV, and in the lpx and pmrA/B genes.

The prevalent mechanism in *A. baumannii* regarding to resistance is the enzymatic degradation by β-lactamases. Taking into account their molecular structure, there are four families of β-lactamases: A, B, C and D or OXA, which includes OXA-23, OXA-24, OXA-51, OXA-143 and OXA-48 (45).

Porins are transmembrane or outer membrane proteins which function is to allow the entrance of antimicrobial agents into the bacteria. Some studies have demonstrated that porins of *A. baumannii* have an important role on the resistance to carbapenems, such as the underexpression of the CarO and Omp33-36 porins which are related to carbapenems resistance (46-48). In addition, loss of OmpA was associated with increase susceptibility of chloramphenicol, nalidixic acid and aztreonam (49).

Efflux pumps are mechanisms which mediate the outflow of toxic compounds for the bacteria, as antimicrobial agents, in a proton coupled exchange. It has been identified in *A. baumannii* five superfamilies of efflux system: ABC, RND, MATE, MFS and SMR (50). The best characterized is AdeABC which confers high resistance to carbapenems.

Aminoglycoside resistance in *A. baumannii* is mainly mediated by the expression of aminoglycoside-modifying enzymes (AMEs), such as acetyltransferases, nucleotidyltransferases, and phosphotransferases (51). The activity of these AMEs results in the modification of the amino or hydroxyl group present in the aminoglycosides which leads to a reduction in their affinity for the target site.

3.2. *Pseudomonas aeruginosa*

P. aeruginosa is an aerobic, rod-shaped, catalase-positive, non-fermentative bacterium, which has unipolar motility. It is found in soil, water, skin flora, hypoxic environments and medical equipment. It is an important cause of infection among patients with impaired immune system.

3.2.1. Clinical and epidemiological importance

P. aeruginosa is a major cause of nosocomial infection, particularly in critically ill, immunocompromised patients, and is associated with greatly prolonged hospitalization, increased costs and high mortality (52, 53).

It causes community and nosocomial infections in patients undergoing invasive procedures in ICUs, with cystic fibrosis, or immunosuppressed (23, 54, 55). This pathogen produces a wide range of infections such as pneumonia, skin and soft tissue infections, urinary tract infection, ocular infection, bacteremia, septicemia and endocarditis (56).

Data provided in 2012 by the ECDC of the ten most frequently isolated microorganisms in ICU infections, showed that *P. aeruginosa* was the first common etiologic agent isolated in ICU-acquired pneumonia episodes in Europe (16.6 %) and in Spain (24.6 %) (3). High percentages of *P. aeruginosa* isolates resistant to aminoglycosides, ceftazidime, fluoroquinolones, piperacillin/tazobactam and carbapenems were reported from several countries. Combined resistance was also common with 14 % of the isolates reported as resistant to at least three antimicrobial classes (3). Furthermore, it has been reported in North America that the mortality rates in VAP and bacteremia caused by *P. aeruginosa* vary between 34-68 %, and between 18-61 %, respectively (57, 58).

3.2.2. Resistance mechanisms

Different antimicrobial resistance mechanisms have been reported with *P. aeruginosa.* As for *A. baumannii*, β-lactamases hydrolysis, porins expression loss and efflux pump overexpression are the principal antimicrobial resistance mechanisms.

P. aeruginosa harbors an inducible AmpC-type cephalosporinase that can be derepressed following mutations in the regulation system (59).

Resistance to tobramycin mostly occurs through the acquisition of AMEs, while resistance to amikacin mostly depends on the overexpression of efflux pumps, such as MexXY (60, 61). Otherwise, fluoroquinolone resistance results from mutations in the topoisomerase-encoding genes and/or the hyper-expression of efflux systems (62). In addition, colistin-resistant mutants of *P. aeruginosa* may emerge in settings with high frequency of colistin use (63).

Furthermore, *P. aeruginosa* has several three-component efflux systems, some of which confer resistance to beta-lactams when strongly expressed after mutations in their promoter regions (64). The most frequently involved system is MexAB-OprM, whose overexpression confers resistance to ticarcillin, aztreonam, cefepime and meropenem. Moreover, with respect to porins modification, it has been demonstrated that the loss of the porin OprD in *P. aeruginosa* manage the passage of imipenem through the outer membrane (62).

3.3. *Escherichia coli*

Enterobacteriaceae is a large family of GNB that includes pathogens as *Escherichia*, *Salmonella*, *Klebsiella*, *Enterobacter*, *Serratia* and *Proteus* between others. They are rod-shaped, facultative anaerobes, sugar fermenting and non-spore forming, and most have many flagella. Many of them are part of our gut microbiota, while others are found in water or soil. In addition, some Enterobacteriaceae produce endotoxins. The most important specie from Enterobacteriaceae with respect to the clinical practice is *E. coli*.

3.3.1. Clinical and epidemiological importance

E. coli is among the Enterobacteriaceae with high clinical interest. This bacterium may cause severe community and nosocomial infections including digestive, urinary tract, intra-abdominal, respiratory, and bacteremia. The 2011 Spanish Nosocomial Infections Prevalence Study (EPINE) showed that *E. coli* is the first (17.9 %) most frequently

isolated pathogen. This situation is similar to that in Europe. The 2013 annual epidemiological report from the ECDC highlights that *E. coli* is the first (15.9 %) most frequently isolated pathogen from HAIs.

Over the last decade, *E. coli* in Europe and worldwide have become increasingly resistant to first and second-line antibiotics (e.g. β-lactams, fluoroquinolones and aminoglycosides). Resistance to extended-spectrum β-lactam antibiotics due to the production of extended spectrum β-lactamases (ESBLs) continued to increase between 2008 and 2011 from 18 % to 28 % for *E. coli*, and show a clear north to south gradient with the highest percentages of resistance reported from Southern Europe (65)**.** One of the most important problems with the antimicrobial resistance in Enterobacteriaceae is the resistance to carbapenems, because of its rapid increase in the last years and by the MDR in these strains, making difficult to have an appropriate therapy for the severe infections caused by them. Resistance to carbapenems in *E. coli* is linked either to decreased permeability or to the enzymatic breakdown of the antibiotic by carbapenemases. The most frequent carbapenemases in these pathogens reported in Europe and worldwide are NDM-1, and OXA-48 (66). With the current increase in the use of colistin, due to the carbapenems resistance, the presence of colistin-resistant *E. coli* has been also reported worldwide (67, 68), in fact, it has extended its geographic distribution, reaching 27 countries (69). Several other studies have reported MDR *E. coli* clinical isolate resistant to colistin and carbapenems (68). Report of the worldwide SENTRY Antimicrobial Surveillance Program showed that this resistance rate has been increased during time.

In United States, an estimated 140,000 healthcare-associated Enterobacteriaceae infections occur each year; about 26,000 and 9,300 of these are caused by ESBLs producing Enterobacteriaceae (EPB) and carbapenems-resistant Enterobacteriaceae (CRE), respectively. Up to 57 % and 50 % of all bacteremia caused by EPB and CRE resulted in death, respectively (70). In Spain, analysis of 4758 *E. coli* bacteremia episodes collected through a blood culture surveillance programme from 1991 to 2007 showed a mortality of 9 % (71). This figure was higher in a Spanish multicentre cohort study, with a mortality rate by ESBL producing *E. coli* and *Klebsiella* spp. bacteremia of 21 % (72). In our centre (University Hospital Virgen del Rocío, Microbiology

Service), during 2015, 323 bacteremia episodes were caused by *E. coli*, which represent the 43.18 % of all GNB that caused bacteremia.

3.3.2. Resistance mechanisms

Recent years have seen increasing numbers of reports on the acquisition of antimicrobial resistance by *E. coli* strains

Some *Enterobacter* spp. produces β-lactamases in order to inhibit the effect of β-lactams antimicrobials. AmpC is strongly induced by amoxicillin, clavulanic acid, cefoxitin and first-generation resistance, which results in intrinsic resistance (73). It is also typical to found OXA-48-producing Enterobacteriaceae, which hydrolyzes penicillins and carbapenems. Moreover, all Enterobacteriaceae are naturally susceptible to quinolones and fluoroquinolones. High-level resistance emerges after successive chromosomal mutations in the DNA gyrase- and topoisomerase IV-encoding genes (*gyrA* and *parC*, respectively) (74). Regarding to colistin, *mcr* genes harboring plasmid have been associated recently with colistin resistance reported in clinical isolates of *E. coli* (75, 76). Also, colistin resistance has been associated with mutations in genes involved in the outer membrane polarity (63). However, the spread of colistin-resistant Enterobacteriaceae is being increased.

3.4. Pathogenicity and virulence mechanisms of GNB

To cause disease, GNB use their virulence factors to first colonize and then infect the host (77). Different genomic, transcriptomic and proteomic studies or animal models of infection have helped to identify phenotypes or virulence factors that participate in the pathogenesis of GNB. Due to the increase of antimicrobial resistance rates of GNB and the lack of treatments to combat the infections that produce, it is important to identify new virulence factors to characterize the pathogenesis and determine new therapeutic targets that allow the control of the infections.

There are several pathogenicity and virulence mechanisms from GNB, as described below.

3.4.1. Outer membrane proteins

GNB contain a double membrane which serves for protection and provide nutrients for viability. As the first line of contact between the bacteria and its external environment, the outer membrane functions as a selective barrier that prevents the entry of many toxic molecules into the cell, while it allows the entry of nutrients required for cell survival (**Figure 2**). The outer membrane is mainly composed by proteins, called outer membrane proteins (OMPs) or porins, which serve essential functions for the cell, including nutrient uptake, cell adhesion, cell signaling and waste export (78). Moreover, for the pathogenic strains, many of these OMPs also serve as virulence factors for nutrient scavenging and evasion of host defense mechanisms (**Table 1**) (79).

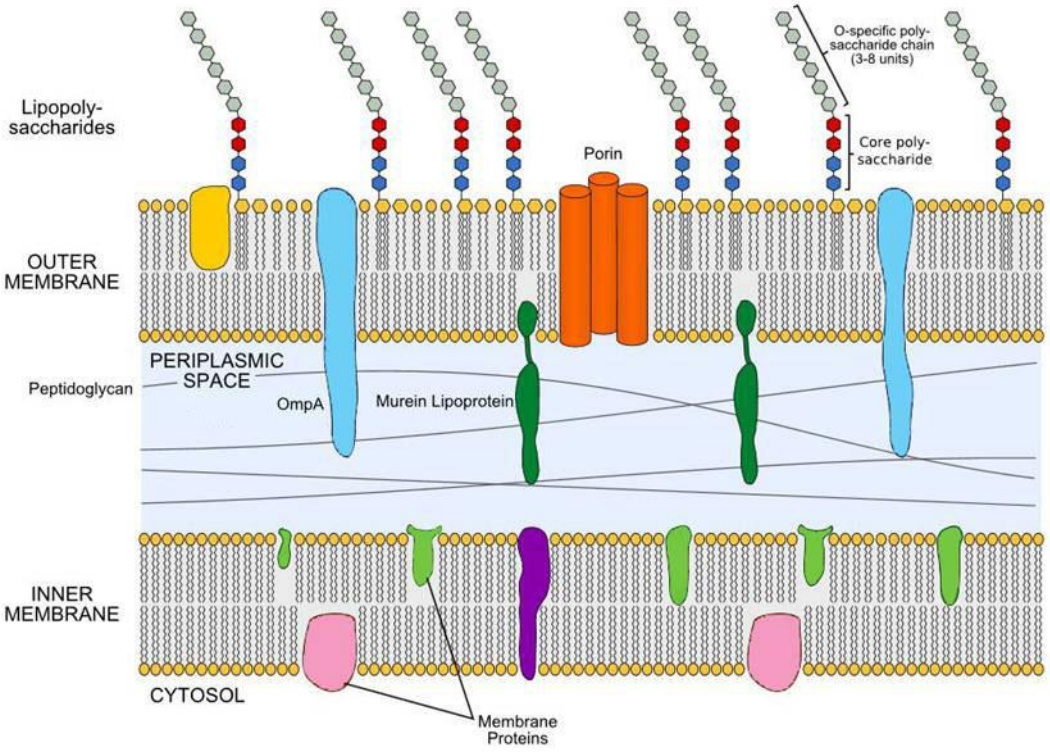

Figure 2. GNB membrane structure.

Among the OMPs, we find OmpA as an important virulence factor of GNB. OmpA, highly preserved among bacterial species, is the most abundant protein on the bacterial surface, which serves a multitude of functions (80). It plays a major role in the adherence and invasion of *A. baumannii* into epithelial cells and induces apoptosis and cytotoxicity, harming the mitochondria and nucleus of eukaryotic cells (81-83). This

protein mobilizes through outer membrane vesicles (OMVs) of *A. baumannii* to reach the host and perform its cytotoxic activity (84). In addition, it is implicated in adherence of *A. baumannii* to host cells (85) and in biofilm formation (81). Furthermore, it has been demonstrated its contribution in the antimicrobial resistance phenotype of *A. baumannii* (49) and its fitness and virulence *in vitro* and *in vivo* (86). Another important OMP is Omp33-36, which induces apoptosis in eukaryotic cells (87) and it is also implicated in bacterial fitness and virulence (88). Furthermore, it has been demonstrated that the lower expression of the porins CarO and OprD-like attenuates the virulence of a PDR *A. baumannii* strain (89).

In *P. aeruginosa*, OprF, an OmpA homologue protein, is required for virulence of *P. aeruginosa* (90). It acts in part through the modulation of the quorum sensing (QS) network (90). Other reports have showed that OprF is involved in the adherence of *P. aeruginosa* to host cells (91). Another important component of the outer membrane component is the MexAB-OprM. It is the most frequent efflux pump which confers resistance to β-lactams (92). In addition, the *P. aeruginosa* porin OprD is a substrate-specific porin that has been shown to facilitate the diffusion of basic amino acids, small peptides that contain these amino acids, and carbapenems into the cells. Its deficiency confers to *P. aeruginosa* a basal level of resistance to carbapenems, especially to imipenem (93, 94).

In *E. coli* it has been evidenced that OMPs are able to promote bacterial resistance to innate immunity, such as OmpA, OmpW, OmpX and OmpF. The importance of OmpA in the pathogenicity of *E. coli* has been established in numerous model systems. OmpA is a virulence factor in meningitic strains of *E. coli* and functions in adhesion to and invasion of central nervous system capillary endothelium and astrocytes (95-97). Weiser and Gotschlich demonstrated that OmpA-deficient mutant of the neurovirulent, meningitic *E. coli* was less virulent in a chick embryo and neonatal rat models and more sensitive to serum complement-mediated bactericidal effects (98). OmpA is also involved in adhesion of enteropathogenic *E. coli* to epithelial cells on mucosal surfaces, leukocytes and macrophages (99). Moreover, OmpA is critical for promoting persistent infection within urogenital epithelium. Nicholson *et al*. have demonstrated that during urinary tract infection, OmpA expression in wild-type bacteria was increased 20-30 fold after infection (100). *E. coli ompA* deficient mutants have lower adherence and invasion

into bladder epithelium than wild-type bacteria (100). In the foodborne enterohemorrhagic *E. coli*, OmpA was demonstrated to be an adhesion molecule for bacterial epithelial cell interaction (101, 102). Double deletion mutants of ompA and tcdA, a transcriptional activator, reduced binding of *E. coli* to plan cells, which are often the source of human infection (103). In addition, OmpA stimulates murine dendritic cells to secrete proinflammatory cytokines IL-1, IL-10 and IL-12 in a dose-dependent and TLR4-independent manner (102).

Table 1. Main outer membrane proteins (OMPs) in GNB pathogenicity.

OMPs	Microorganism	Function
OmpA	*A. baumannii* *P. aeruginosa* *E. coli*	Adherence, invasion Cytotoxicity Biofilm formation Bacterial fitness and virulence
Omp33-36	*A. baumannii*	Apoptosis in eukaryotic cells Bacterial fitness and virulence
CarO	*A. baumannii*	Carbapenem resistance
OprD	*P. aeruginosa*	Carbapenem resistance
OprM	*P. aeruginosa*	β-lactams resistance
OprF	*P. aeruginosa*	Bacterial virulence
OmpF	*E. coli*	Passive diffusion of small molecules
OmpW	*E. coli*	Resistance to phagocytosis
OmpX	*E. coli*	Attachment to abiotic surfaces

3.4.2. Bacterial adherence and biofilm formation

The ability of bacteria to interact with eukaryotic cells is the first step in the pathogenesis process, followed by their internalization into the host. Invasive bacteria

reach a compartment in which they are protected against host clearance mechanisms, can replicate and prepare themselves to gain access to tissues and circulatory system.

There are different structures, from both the pathogen and the host cell, by which bacteria allow its adhesion to the eukaryotic cell. Some of these bacterial components are OmpA, pili and fimbrial-like structures (81, 104, 105). Otherwise, there are also host cell surface factors that can mediate bacterial adherence, such as integrin and fibronectin (85, 106). Furthermore, it has been shown that *A. baumannii*, expressing phosphorylcholine (ChoP) in outer membrane, binds to human lung epithelial cells through platelet-activating factor receptor (PAFR), activating clathrin and β-arrestins to uptake the bacteria (107), and it has been suggested that *A. baumannii* has independent molecular mechanisms to adhere to different surfaces (108).

As the **figure 3** shows, once the first bacteria adhere to the biotic and abiotic surface, the biofilm starts to be formed. Biofilms are surface-attached bacterial communities encased in a matrix of exopolysaccharides, proteins, and extracellular DNA (109), which confer to bacteria a protection mechanism to survive in unfavorable environments and during the infection.

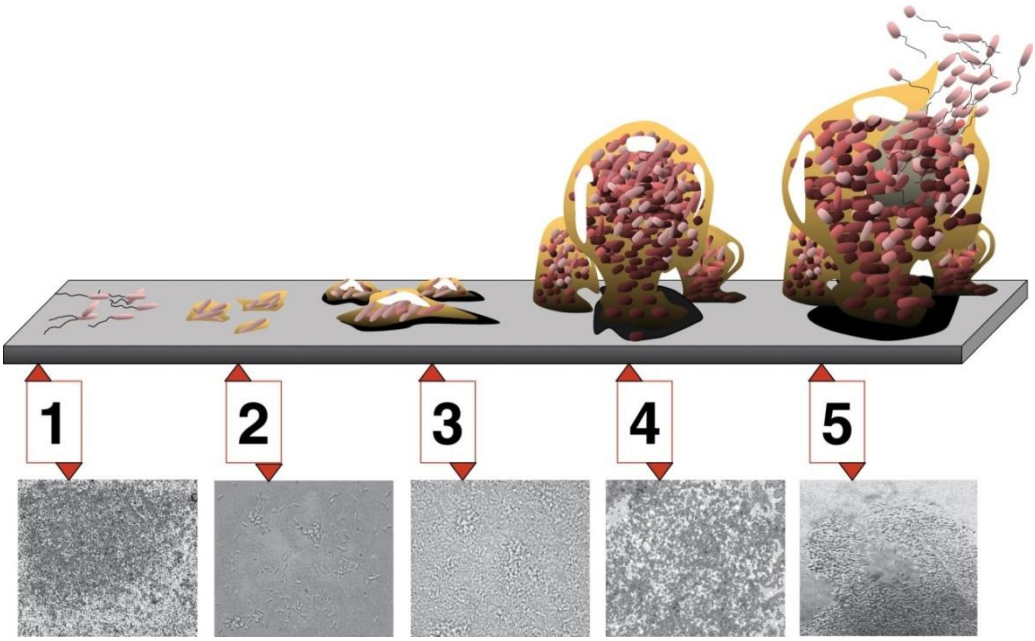

Figure 3. Representation of biofilm formation. The formation of biofilm starts with [1] single free floating bacteria which land on a surface. [2] Then this bacterial cells aggregate and attach to the surface, and [3] growth and divide for biofilm formation. [4] Represents a mature biofilm formation, and [5] part of biofilm disperses to release free floating bacteria for further colonization.

The matrix material, although is produced by the individual cells, forms structures that provide benefits for the entire community, including protection of the cells from various environmental stresses (110), and does not allow the antibiotic to be efficient against bacteria (111). Moreover, biofilms are complex and dynamic (112), because biofilm cells are not static and are heterogeneous. This ability allows the bacteria to colonize hospital equipment (113) and also biotic surfaces, as epithelial cells (114).

It has been demonstrated that some genes are implicated in biofilm formation. The two-component regulatory systems and transcriptional regulators related to the genes associated with biofilm are very important (115). The most important gene in *A. baumannii* is *CsuE*, which is the component of the CsuA/BABCDE chaperone-usher complex. This gene is involved in the production of the pili, and when the inactivation of the gene is produced no biofilm formation is observed (116). It has been also describe in *A. baumannii* that the outer membrane protein A (OmpA) participates in biofilm formation is plastic surfaces, and that it is essential for the adhesion to human lung epithelial cells (81). In *P. aeruginosa* there is one gene cluster called chaperone-usher pathway A (*cupA*), which is required for biofilm formation on abiotic surfaces, and it has been determined that is regulated by the transcriptional regulator MvaT, controlling biofilm formation and maturation in *P. aeruginosa* (117). For *E. coli*, it has been established that the RNA binding global regulatory protein CsrA serves as both a repressor of biofilm formation and an activator of biofilm dispersal under a variety of culture conditions (118).

3.4.3. Motility

To move actively over surfaces, bacteria employ several behaviors, showed in **figure 4**, as swarming, swimming, twitching, gliding and sliding (119). Swarming and twitching, the main motility activities, occur on soft and moist surfaces, but swarming is usually propelled by the rotation of flagella (120), and twitching is powered by the extension of type IV pili. (121). Swimming is the movement of individual bacteria in liquid, powered by rotating flagella. Gliding is active surface movement that does not require flagella or pili and involves focal-adhesion complexes. Sliding is passive surface translocation that is powered by growth and facilitated by a surfactant (119).

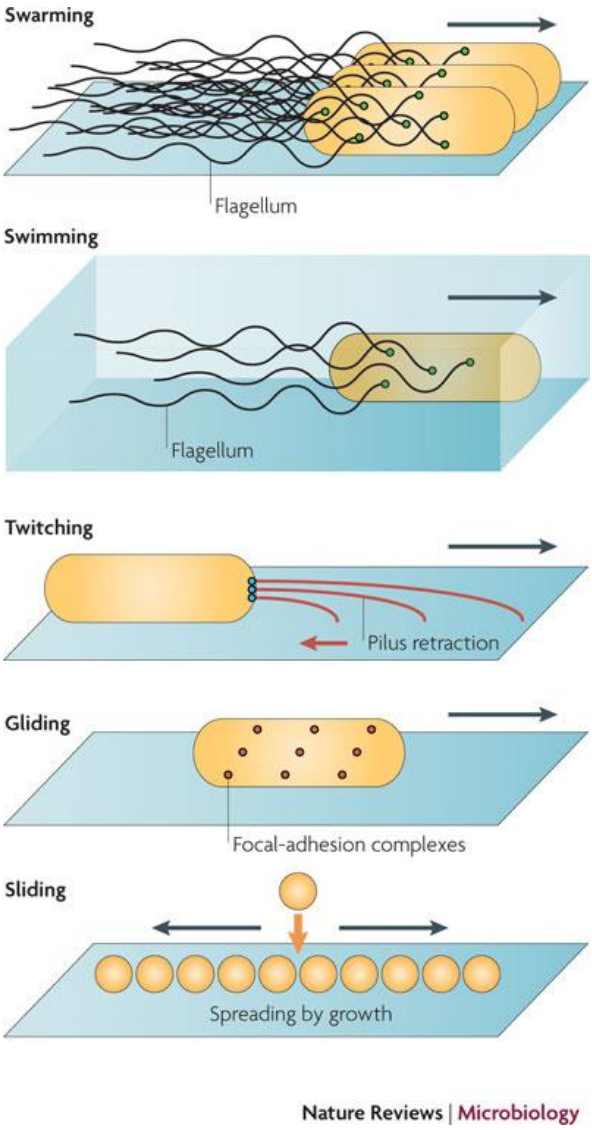

Figure 4. The diverse ways by which prokaryotes move. Figure taken from **Jarrell KF**. Nat Rev Microbiol. 2008. 6(6):466-76.

P. aeruginosa possess a polar flagellum and multiple type IV pili (122), thus it can move by swarming or twitching motility. During swarming, *P. aeruginosa* retains its polar flagella but synthesizes an alternative motor specifically required to move on surfaces and through viscous environments (123). In contrast, *A. baumannii* does not have a flagellum, and it has been demonstrated that it has twitching motility (124, 125). With respect to *E. coli*, it uses multiple flagella oriented axially along the cell body to propel cells through bulk liquids (126).

3.4.4. Surface lipolysaccharides

Lipopolysaccharide (LPS), which is present in the outer membrane of the GNB, plays an important role in their virulence. It is composed by three structural domains: a hydrophobic domain known as lipid A (or endotoxin), a core oligosaccharide and a highly variable O-antigen constituted of repeating oligosaccharide units (127).

It has been demonstrated its lethal toxicity in mice, pyrogenicity in rabbits and complement inactivation *in vivo* (128). Furthermore, LPS activates the innate immune response (129). Otherwise, it is known that colistin resistance in GNB is most common due to LPS loss or modification, caused by changes in PhoPQ and PmrAB, which results in a less anionic lipid A (77, 130-132).

3.4.5. Outer membrane vesicles

Outer membrane vesicles (OMVs) are spherical nanovesicles, with a diameter of between 20 and 200 nm, of the outer membrane filled with periplasmic content, and are commonly produced by GNB (133). Their composition is DNA, RNA, lipids, LPS and even OMPs (84, 134). These vesicles can transport virulence factors, such as OmpA (84); participate in biofilm formation, and may also be involved in quorum sensing and antimicrobial resistance gene transfer (135). Therefore, OMVs have high relevance not only because of their importance in the host-pathogen interaction, but also because of their ability to disseminate resistance genes.

3.4.6. Siderophores

Siderophores are low molecular weight, high affinity iron chelating molecules that are essential factors in many GNB. Their function has been defined as the chelation and delivery of iron to bacteria for proliferation (136). It is well known that iron is essential for the growth of bacteria, therefore these microorganisms have developed highly efficient iron-acquisition systems, as siderophores to obtain iron from outside of the bacteria (137). It has been identified different siderophores, as acinetobactin, achromobactin and fimsbactin A-F from *A. baumannii;* ferrichrome, pyoverdines and pyochelin from *P. aeruginosa;* or enterobactin and aerobactin from *E. coli* (137).

3.4.7. Quorum sensing

The quorum sensing (QS) is a mechanism from bacteria to communicate cell-to-cell, excreting chemical compounds (138). It is used by bacteria to coordinate the expression of several collective traits, including the production of multiple virulence factors, biofilm formation and motility once a population threshold is reached (139). QS depends on the production, secretion and detection of small diffusible autoinducers, such as acyl-homoserine lactones (AHLs), auto-inducing oligo-peptides (AIPs) and autoinducer 2 (AI-2). AHLs are produced in *A. baumannii* and *P. aeruginosa* by the LuxI family that interacts with LuxR (140), and AI-2 is produced by *E. coli* (141).

There are *in vitro* and *in vivo* evidences that link QS systems with human infections (142). Moreover, it has been suggested that QS and MDR regulatory systems might have some evolutionary relationship and biological relevance; because antibiotics also act as signal molecules, and the efflux pumps for antibiotics are also the exporters for QS signals (143).

4. Current treatment and drugs options

The rising predominance of HAIs caused by MDR bacterial pathogens is restricting the options for effective antimicrobial therapy. As previously detailed, this worrying spread of antimicrobial resistance has not been extending equally to the development of new antimicrobials. In this context, the reintroduction of previously used antibiotics, alone or in combination, has emerged as a new strategy to deal with infections caused by MDR strains (144, 145). A list of antimicrobial agents used currently in clinical setting is described as following.

4.1. Cephalosporins

Cephalosporins are among the most widely used antibiotics worldwide due to their wide spectrum of activity, good pharmacokinetics, established clinical efficacy and high tolerability. The cephalosporins interfere with cell-wall synthesis of bacteria, crossing

the bacterial cell wall and binding to the penicillin-binding proteins, which leads to lysis of the infectious organism (146).

The cephalosporins are separated into 4 classes of generations according to their spectrum of activity. The third and fourth generations are developed successively, in the 80s and 90s respectively. The third-generation of cephalosporins includes cefoperazone, cefotaxime, ceftazidime, ceftizoxime, ceftriazone and moxalactam (146). These drugs have been important antimicrobial agents in the treatment of nosocomial infections. The fourth-generation of cephalosporins include cefpirome and cefepime, which have a more balanced antimicrobial spectrum of activity against Gram-positive and Gram-negative microorganisms compared to the third-generation of cephalosporins (147).

Ceftazidime has an *in vitro* broad spectrum activity against Gram-positive and Gram-negative aerobic bacteria (148). Ceftazidime's primary advantage over other cephalosporins is that it has broad-spectrum activity against GNB including *P. aeruginosa* (149). Because of its superior antipseudomonal activity, ceftazidime is frequently used for empiric therapy in neutropenic patients with unexplained fever. Furthermore, it has excellent penetration into the cerebrospinal fluid and may, therefore, be useful against *P. aeruginosa* meningitis, and is also used in treating meningitis caused by GNB such as *E. coli* and by *Klebsiella* spp. (146)**.**

Resistance to cephalosporins results from a variety of mechanisms: β-lactamase production, alteration of penicillin-binding proteins, and alteration of the cell-wall permeability of GNB (146).

4.2. Carbapenems

Carbapenems are considered to have the most potent and widest spectrum of antimicrobial activity among the different classes of β-lactams. Their spectrum of antimicrobial activity includes Gram-positive and Gram-negative aerobic and anaerobic pathogens (150). Nevertheless, to provide optimal bactericidal exposure and minimize the development of drug resistance, it has been prompted the consideration of alternative dosing strategies, such as prolonged infusion, higher doses, and combination therapy (151).

Imipenem was the first carbapenem released for clinical use and has the broadest antibacterial activity, high potency, and no cross-resistance with other β-lactam antibiotics. It is active against *A. baumannii*, *P. aeruginosa*, *Enterobacteriaceae*, streptococci, methicillin-sensitive staphylococci, *Neisseria*, *Haemophilus* and anaerobes (152-154).

The imipenem mechanism of action consists on binding to bacterial penicillin-binding proteins, which are responsible for elongating and cross-linking the peptidoglycan of the bacterial cell wall. Consequently, the construction of the cell wall is impaired causing inhibition of cell growth, cell lysis and death (155).

The broad spectrum of antimicrobial activity of imipenem is thought to be attributable to three factors: (i) its ability to penetrate the cell membrane of multiple GNB through porins due to its smaller size, (ii) its affinity for penicillin-binding proteins (peptidases) from a broad ranges of bacteria, and (iii) its resistance to a broad range of β-lactamases from Gram-positive and GNB (156).

It has been reported the emergence of resistance to imipenem during treatment against *A. baumannii* and *P. aeruginosa* infections through alteration in permeability of the cell membrane, mutation in PBPs and hydrolysis by carbapenemases (157). In addition, it has been described that carbapenems resistance in *E. coli* was associated with the simultaneous loss of the two major porins, OmpF and OmpC, and with plasmid-mediated AmpC and ESBL production (158).

4.3. Tigecycline

Glycylcyclines, discovered in 1993, are structural analogues of tetracycline designed to avoid resistance mediated by efflux pumps and ribosomal protection (159). Glycylcyclines exhibit antibacterial activities typical of earlier tetracyclines, but with higher activity against tetracycline-resistant microorganisms (160). Tigecycline is the first glycylcycline which received approved labeling from the Food and Drug Administration (FDA) in June 2005. It has been approved for the treatment of complicated skin and skin structure infections and intraabdominal infections in patients over 18 years of age (161).

Tigecycline represents an effective option to treat infections caused by a variety of aerobic Gram-positive and Gram-negative pathogens, as MDR *A. baumannii* infections (162) and *E. coli* (163), but lacks activity against *P. aeruginosa*, and its use has not been validated for all organ-specific infections (164).

Regarding its mechanism of action, tigecycline enters bacterial cells through energy-dependent pathways or with passive diffusion, and reversibly binds to the 30S subunit of the ribosome. It acts by blocking the incorporation of transfer RNA into the A site of the ribosome, thus prevent the incorporation of amino acids residues into elongating peptide chains, inhibiting protein synthesis (165, 166).

Tigecycline is not affected by most of the common mechanisms of antibiotic resistance used by bacteria to circumvent antibiotic therapy. Tigecycline resistance mechanisms of major clinical importance include the major facilitator superfamily (MFS) efflux pumps and ribosomal protection proteins (167). The adeABC-FGH-IJK efflux pumps genes in *A. baumannii* are regulated by the adeRSL regulatory genes and mutations in these genes confer resistance to tigecycline trough overexpression of efflux pumps (168, 169). For instance, previous results from our group revealed the presence of the gene *tetX* in *A. baumannii* plasmid, which is correlated with the increase of tigecycline MIC in *A. baumannii* (170). Intrinsic resistance to tigecycline has been reported in *Pseudomonas* spp. (171). In *P. aeruginosa* the efflux system ABCDXY-oprJM is unique and is regulated by the *mexR*, *mexZ* and *nfxb* regulatory genes that repress the overexpression of these efflux pumps, conferring susceptibility in wild type strains and resistance in strains bearing mutations in these structural and repressor genes (172). In *Enterobacteriaceae*, resistance to tigecycline involves upregulation of some efflux pumps, such as AcrAB-TolC pump and its regulator ramA (173). Specifically, in *E. coli*, tigecycline is a possible substrate for AcrAB and AcrEF (174). Moreover, the *mar* regulon induces the downregulation of the OmpF outer membrane porin and the overproduction of the AcrAB-TolC efflux pump, and the increased expression of MarA and AcrAB has been reported in strains with elevated tigecycline MICs (171).

4.4. Colistin

Colistin, also called polymyxin E, is a lipopeptide isolated from *Bacillus polymyxa*. The polymyxins group, discovered in the 1940s, was among the first antibiotics with significant activity against GNB (175). There are five types of polymyxins (A-E), but only polymyxin B and E have been used in humans (164). However, in the 1970s they were largely replaced by other antibiotics due to its nephrotoxicity and neurotoxicity (176). Although the toxicity of colistin was re-evaluated by some researchers who found that the incidence of toxicity is less frequent and severe compared with what has been previously reported (177).

The high prevalence of infections by MDR isolates has led to their reconsideration as a therapeutic option that has promoted an increase in its use (164, 178). Colistin is only active against MDR GNB, including *A. baumannii*, *P. aeruginosa* and *Enterobacteriaceae* (179, 180).

Regarding the mechanism of action, colistin has a strong positive charge and a hydrophobic acyl chain that give it a high binding affinity for LPS molecules. Colistin interacts electrostatically with LPS, displacing divalent cations from the molecule, causing disorganization of the outer membrane (181). As a result, the permeability of the cell envelope is increased, causing leakage of cell contents and, subsequently, cell death (182). Colistin also binds to the lipid A portion of LPS and, in animal studies, block many of the biological effects of endotoxin (183)

Unfortunately, colistin resistance has emerged recently worldwide especially among patients treated with colistin, even though still relatively low (184). Due to this fact, some researchers have concluded that acquisition of colistin resistance in patients is due, among others, to the colistin therapy and nosocomial transmission of colistin resistant strains (185). Nevertheless, it has been also reported the *in vivo* emergence of colistin resistance in humans without colistin therapy or clonal transmission (186).

A. baumannii acquires resistance to polymyxins by the modification of the lipid A moiety of LPS, resulting of mutations in the pmrA/pmrB two-component system, or by the complete loss of LPS caused by either mutations or the insertional inactivation of lipid A biosynthesis genes (63). As in *A. baumannii*, *P. aeruginosa* is able to acquire

colistin resistance by the mutation in the pmrA/pmrB system, as well as the phoP/phoQ system (63).

In *E. coli*, Liu *et al*. have described recently the gene *mcr-1*, carried on a plasmid with high conjugation efficiency, which codes for a phosphoethanolamine transferase enzyme that catalyzes a change in the colistin target conferring resistance (67). In addition, this gene and its variant *mcr-2* have been detected in isolates recovered from food animals around the world and from human patients (68, 187-189). This implies that the evolution of colistin resistance in bacteria could be more complex than previously thought.

4.5. Combination antimicrobial therapy

An approach which is being used to treat severe Gram-negative infections is the combination antimicrobial therapy. The combination treatment with different antimicrobials is recommended for severe sepsis, septic shock and pneumonia caused by GNB to reduce mortality related to inappropriate antibiotic treatment (190, 191). The use of combination therapy as the treatment for Gram-negative infections is justified by one of these reasons: (i) to broaden the empiric coverage provided by two antimicrobial agents with different spectra of activity, (ii) to achieve the synergy observed *in vitro* between two antibiotic agents compared to monotherapy, (iii) to prevent or delay the emergence of resistance during antimicrobial therapy (191, 192)**.**

The mechanisms of synergy are often not fully understood, but there are some explanations for a few antibiotics. For instance, colistin, which is frequently a component of effective combinations, increases the permeability of other antibiotics through the bacterial outer membrane by a detergent mechanism (151). This mechanism can balance acquired resistance mediated by decreased antibiotic permeability (e.g. porin loss), and will also empower antibiotics that are not traditionally considered appropriate treatment options for GNB. For example, the addition of rifampin to colistin and meropenem/doripenem has resulted in synergistic effects *in vitro* against MDR *Acinetobacter* spp., *Pseudomonas* spp., and carbapenemase-producing *Enterobacteriaceae*, and has been described as successful treatment in previous reports (193-196).

Moreover, with respect to colistin, both *in vitro* and *in vivo* combination tests have been performed to treat *A. baumannii* infections (197, 198). Colistin plus imipenem combination has been confirmed that is synergistic against heteroresistant isolates and prevents the development of colistin-resistant mutants *in vitro*, as well as colistin with rifampin (199), which has also shown efficacy in experimental models of pneumonia and meningitis (200). Furthermore, the combination of colistin with tigecycline showed favorable results *in vitro* against tigecycline non susceptible isolates (201). Against MDR *P. aeruginosa*, synergistic effects have been demonstrated for double and triple antibiotic combinations including an aminoglycoside, an anti-pseudomonal beta-lactam, colistin, a fluoroquinolone, a macrolide, or rifampin (195, 202). Combinations of colistin with tigecycline or with a carbapenem have been advocated for carbapenemase-producing *Enterobacteriaceae* (203, 204) and even double and triple antibiotic combinations that include an aminoglycoside, aztreonam, a carbapenems, colistin, rifampin, tigecycline, or fosfomycin have demonstrated synergistic or bactericidal effects *in vitro* (195).

Nevertheless, there is evidence that the approach of combination therapy may be harmful. It might be associated with increased risk of toxicity, selection of resistant strains and superinfection (205). Furthermore, clinical data to support the alternatives of combinations are insufficient (190).

5. Novel therapies against GNB

5.1. New molecules and adjuvants for infections treatment

Antibiotic resistance is a ubiquitous and relentless clinical problem that is compounded by a lack of new therapeutic agents. The retreat of the pharmaceutical sector from new antibiotic development has exacerbated the challenge of widespread resistance and signals a critical need for innovation such as non-antimicrobial approaches. All these reasons have made necessary the urgent search for new alternatives for the treatment and control of infections by GNB. Not killing bacteria but avoiding the infection produced by GNB, either immunizing the host or blocking the bacterial virulence factors, could be adjuvant approaches to reach new therapeutic goals. Another approach would be to enhance the antibiotic activity in MDR and XDR GNB infections

administering antibiotics in conjunction with adjuvants (non-antibiotic) compounds (206). Thus, it is worthwhile to analyze the main results on the development of adjuvant therapeutics as monotherapy or in combination therapy in order to restore the efficacy of currently used antibiotics in the clinical setting.

5.1.1. Adjuvants drugs in monotherapy

5.1.1.1. Adjuvants with immunomodulatory properties

The innate immune response is very important to control bacterial infections and their clinical outcomes. Some studies have been focused on the stimulation of the immune system by different approaches in order to investigate the role of adjuvants as potential treatments against GNB infections.

a. Lysophosphatidylcholine

Lysophosphatidylcholine (LPC) is a major component of phospholipids in eukaryotic cells. It is implicated in immune cell recruitment and modulation (207) as monocytes, phagocytes and T lymphocytes (208). It has been described that LPC protected mice against lethality after cecal ligation and puncture or intraperitoneal injection of *E. coli* (209), and after development of peritoneal sepsis by *A. baumannii* (210). LPC markedly upgrade spleen and lung bacterial clearance and reduced bacteremia, presumably due to the modulation of immune response (210).

b. Antimicrobial peptides

Other approaches to modulate the immune system are the use of small peptides to control the infections caused by GNB.

For instance, skin secretions from many species of Anura (frogs and toads) contain cytotoxic peptides that are an important part of the innate immune response and provide the first line of defense against invading microbial pathogens (211, 212). These peptides are multifunctional, possessing immunomodulatory and chemoattractant properties as

well as cytotoxic activities, so that it is more informative to refer to them as host-defense peptides rather than exclusively as antimicrobial peptides (213-215).

Between these host defense peptides, which are an evolutionarily conserved components of the innate immune system among most multicellular organisms (216), we can found the hymenochirin-1B, the apolipoprotein E (apoE) and Esculentin-2CHa.

The hymenochirin-1B displays moderate growth-inhibitory activity against reference strains of GNB and possesses relatively low hemolytic activity against human erythrocytes (217). Besides, it was developed its non-toxic analog [E6k,D9k]hymenochirin-1B, which also has higher potency over the native peptide against clinically relevant microorganisms as *A. baumannii*, *K. pneumoniae* and *E. coli* (218).

With respect to ApoE, it is an important protein that is involved in the metabolism and transportation of lipids, as well as in the immune response (219). It has been identified an apoE mimetic peptide analogue of the receptor-binding region of apoE (apoE23) which presents immunomodulatory properties *in vitro*, downregulating the expression of IL-6, TNF-α and IL-10 in LPS-induced THP-1 cells, and also shows high levels of selective antibacterial activities against MDR *A. baumannii*, *P. aeruginosa*, and *E. coli*, (220).

The peptide Esculentin-2Cha was developed and shows potent growth-inhibitory activity against reference strains of *P. aeruginosa*, *E. coli*, and *K. pneumoniae* (221) and MDR clinical isolates of *A. baumannii*. It stimulates the release of IL-10 by mouse lymphoid cells and TNF-α by peritoneal macrophages (222). In addition, treatment with the proline-rich antibacterial peptide A3-APO protected mice against systemic infection caused by MDR *A. baumannii*, in spite of its high MIC against this pathogen (223, 224), and upregulated the anti-inflammatory cytokines IL-4 and IL-10 (225).

In the case of peptides of the human innate immune system, beta-defensins hBD-2, hBD-3, and hBD-4 have presented bactericidal activities against MDR *A. baumannii* and *P. aeruginosa* (226-229). LL-37, a cationic peptide of the cathelecidins family, has shown to exhibit significant antimicrobial activity against MDR and colistin-resistant *A. baumannii* strains and prevented biofilm formation, adding significance to its efficacy (230, 231).

c. Small molecules

The immune system can be modulated not only using peptides, but also small molecules, such as the 3',5'-cyclic diguanylic acid (c-di-GMP). This small molecule presents an important signaling molecule that is emerging as an universal bacterial second messenger in regulating bacterial growth on surfaces (232). It stimulates the innate and adaptive immunity (233, 234) and provides a protective effect against several bacterial infections in mice (235).

d. Inhibition of LPS

The inhibition of the bacterial synthesis of LPS through the blocking of *lpx* genes could be a potential adjuvant treatment approach (236). LpxC inhibition by LpxC-1 blocks LPS biosynthesis, which has no effect on *A. baumannii*, but rather enhances phagocytosis and decreases inflammation, resulting in protection of mice from lethal infection (236). In this study, no cross-resistance to the LpxC-1 was reported. Advancement of novel classes of LpxC inhibitors has been performed and LpxC-2, LpxC-3, and LpxC-4 inhibitors were developed to characterize a lead compound, LpxC-4, in terms of its microbiological spectrum, resistance potential, and *in vivo* efficacy (237-239). It has been demonstrated that LpxC-4 spectrum of activity extends to several GNB such as *A. baumannii*, *P. aeruginosa*, and members of the *Enterobacteriaceae*, as *E. coli* and *K. pneumoniae* (239). In a similar strategy, the LpxA and LpxD structures have been recently crystallized and they will be tested as targets for new antibacterial agents (240, 241). Moreover, the synthetic cyclo-peptide antibiotic POL7080, whose activity is limited to *P. aeruginosa*, targeting the *Pseudomonas* LPS-assembly protein (LptD), is currently on clinical trial, on the phase II of development (242).

5.1.1.2. Adjuvants without immunomodulatory properties

The effects and/or antibacterial activity against GNB of other peptides and small molecules without immunomodulatory properties have also been evaluated, both *in vitro* and *in vivo* using experimental models.

a. Antimicrobial peptides

In this category of adjuvants, we can find Cecropin A-melitin hybrid peptides that were evaluated *in vitro* and *in vivo*. They have good *in vitro* activity against colistin-resistant clinical *A. baumannii* strains (243, 244), but in a sepsis model of infection by PDR *A. baumannii*, these peptides have shown short-term efficacy due to their possible lysis by serum proteases (245).

The mastoparan family is considered as a very promising group of potential new antimicrobial peptides drugs (246). Different studies have shown *in vitro* that mastoparan and three of its analogs may be potential antibacterial agents to treat infections caused by MDR and PDR *A. baumannii* (247, 248). In fact, mastoparan analogs, showed the same activity against *A. baumannii* as the original peptide (2.7 μM), maintained their stability in the presence of human serum for more than 24 hours compared to the original compound and showed moderate toxicity in HeLa cells.

An α-helical porcine myeloid antibacterial peptide, PMAP-23, shows killing activity against a broad spectrum of microbial organisms, such as *E. coli*, via the interaction with outer membrane containing LPS (249). In addition, PR-39 which was isolated from the small intestine of the pig penetrates the outer membrane and rapidly kills growing *E. coli* cells via a mechanism that stops protein and DNA synthesis (250).

Some non-α-helical peptides isolated from amphibians, such as esculentin 1bEsc(1-18) and bombinin H2 showed *in vitro* bactericidal activity against clinical isolates of MDR *A. baumannii*, *P. aeruginosa* and *Enterococcus faecium* (251). Moreover, antimicrobial peptides isolated from spider venom, as lycosin-I, presented high antibacterial activities and rapid bactericidal effects against 18 MDR *A. baumannii* strains (252). Regarding the therapeutic efficacy of antimicrobial peptides in experimental models of infection, we can find that intravenous injection of 6 mg/kg K_6L_6 peptide reduced the mortality of neutropenic mice infected with gentamicin-sensitive *P. aeruginosa* and gentamicin-resistant *A. baumannii* clinical strains (253). Although their data suggest that the protection of the mice was due to killing of the bacteria, previous studies have shown that positively charged antimicrobial peptides can bind to LPS and neutralize the LPS-stimulated inflammatory response by macrophages (254). Besides, an α-helical peptide, $C(LLKK)_2C$, showed a high reduction in the tissue bacterial burden and mortality of

immunosuppressed mice, in mouse models of peritonitis and pneumonia infections caused by carbapenems-resistant *A. baumannii* (255).

b. Small molecules

The small molecule BAS00127538, which is an inhibitor of the precursor in cell wall biosynthesis lipid II, was found to affect cell wall biosynthesis with membrane perturbation (256). BAS00127538 has shown an *in vitro* activity, MIC of 2–8 μg/mL, against colistin susceptible and resistant *A. baumannii* isolates, regardless of their antimicrobial resistance profile (257).

A strategy to identify novel treatment targets and antimicrobial molecules has been developed, such as the compound 14, which simultaneously suppresses antibiotic resistance and virulence of *P. aeruginosa* by reducing biofilm formation and bacterial colonization in the acute mouse pneumonia model (258). Another study had identified a series of highly effective small molecules that suppress *P. aeruginosa* redox-active virulence factor by inhibition of the production of pyocyanin (259).

In the case of *E. coli*, a small-molecule library was screened to identified active compounds against this pathogen, resulting in two compounds (5175178 and 5215319) that exhibited antibacterial activity co-related with GlmU inhibition, which is involved in the cell wall biosynthesis of GNB microorganisms (260).

Otherwise, bacterial type I signal peptidases, which are required for the bacterial metabolic process, represent a highly conserved and essential target for inhibition by novel compounds. An inhibitor of these signal peptidases, MD3, has demonstrated potent activity against susceptible and MDR *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*, with reduction in the viable ranging from 70 % to 97 % after exposure to 28 μg/ml of MD3 in the case of *A. baumannii* (261).

c. Antibiofilm drugs

Although there are only a few studies, the activity of antibiofilm drugs has been assessed. DispersinB, an another antibiofilm enzyme produced by the oral bacteria *Aggregatibacter actinomycetemcomitans*, alone or coupled to KSL-V peptide, inhibited biofilm formation and dispersed preformed biofilm in chronic wound infection by *A. baumannii* without affecting bacterial growth (262, 263). In addition, 2-aminoimidazole (2-AI) compound which targets the response regulator BfmR, inhibits and controls biofilm development in *A. baumannii* (264, 265) that has been shown to be a master controller of biofilm formation in *A. baumannii*.

Furthermore, antibiofilm peptide 1018 was shown to inhibit biofilm formation and eradicated preformed biofilms formed by MDR Gram-negative pathogens (266). In *P. aeruginosa*, the peptide 1018 (VRLIVAVRIWRR-NH2) disperses mature biofilm formation at low concentrations (0.8 μg/mL) and kills biofilm cells at higher concentrations (10 μg/mL) (266). Besides, four chimeric peptides have prevented biofilm formation by *A. baumannii* clinical isolates, and exhibited significant antibacterial effects (MIC = 3.12 to 12.5 μM) better than ampicillin, cefotaxime, ciprofloxacin, tobramycin, and erythromycin (267).

In addition, antibiofilm activity by *P. aeruginosa* has been displayed by the THR-SK010 ethanol extract from herbal recipes, suggesting further investigation to explore its possible utilization as an antibiofilm agent, especially for wound treatment (268).

In *E. coli*, indole, which is generated by the degradation of tryptophan by tryptophanase, has the potential to inhibit biofilm formation in several clinical relevant bacterial strains (269). In this pathogen it has been also tested the compound β-sitosterol glusoside, which was identified as a potent inhibitor of *E. coli* biofilm formation trough *rssAB* and *hns* mediated repression of flagellar master operon *flhDC* (270). Moreover, in another study, gallium nitrate was demonstrated to inhibit the growth and auto-aggregation of *E. coli* on titanium (271).

d. Other compounds

Ceragenins, also called cationic steroid antimicrobial (CSA) that were designed to mimic the activities of antimicrobial peptides but are not peptide based (272), were developed to mimic antimicrobial peptides. Among them, CSA-13 showed *in vitro* activity against colistin-resistant *A. baumannii* and *P. aeruginosa* (273, 274), and carbapenem-resistant *A. baumannii* (275). The *in vitro* synergistic activity of ceragenin CSA-13 in combination with colistin, tobramycin and ciprofloxacin against 60 carbapenem-resistant *A. baumannii* bacteremic strains demonstrated synergistic interactions with all the tested combinations. Mostly, these synergistic effects are observed with CSA-13 plus colistin (55 %), whereas the least synergistic activity was observed with CSA-13 plus tobramycin (35 %) (275). A recent study has shown that the ceragenin CSA-131 has *in vitro* activity against colistin resistant *A. baumannii* and *P. aeruginosa* (MIC_{90} 2 μg/mL) better than CSA-13 (276). CSA-131 and CSA-13 have the same scaffold, being the only modification in CSA-131 the length of the aliphatic tail with just one carbon difference (276).

Eventually, blocking the iron acquisition by gallium nitrate [Ga $(NO_3)_3$], the active component of an FDA-approved drug (Ganite), reduced the bacterial *in vitro* growth of MDR *A. baumannii* and *in vivo* reduced the bacterial lung burden in mice and increased the survival of *Galleria mellonella* larvae infected by MDR *A. baumannii* (277, 278).

The bactericidal activity of some flavonoids isolated from *Artocarpus* spp. has been tested for GNB. For instance, only artocarpin showed some antibacterial activity against *P. aeruginosa* with a MIC value of 286.4 μM. In contrast, artocarpin and artocarpone showed antibacterial activity against *E. coli* with MICs values of 71.6 μM and 12.9 μM, respectively (279). Essential oil components, such as hibiscuslide C, also show antibacterial activity in *P. aeruginosa*, related to DNA fragmentation and damage (280), or like α-terpineol, which inhibits *E. coli* growth at ultrastructural level, decreasing cell size, disrupting cell wall and cell membrane and inducing an irregular cell shape (281).

5.1.2. Adjuvant drugs in combination therapy

The efficacy of the adjuvants drugs would be improved by combining them with the classical antimicrobial approach, currently used in the clinical setting. Consequently, an

increasing number of studies describe the combination of different adjuvants, such as peptides or small molecules, *in vitro* and *in vivo*, to face the scarce alternatives of the antibiotics to fight against infections caused by MDR pathogens.

a. Antimicrobial peptides

The need to develop new approaches to treat MDR GNB severe infections has prompted the *in vitro* and *in vivo* evaluation of an important number of antimicrobial peptides, including synthetic and natural. Different studies have evaluated the possible synergy between peptides and clinically used antimicrobials.

The antibiofilm peptide 1018, with *in vitro* activity alone, has shown also synergism activity with ciprofloxacin, ceftazidime, imipenem or tobramycin decreasing by 2 to 64 fold the concentration of antibiotic required to treat biofilms formed by *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* (282).

A third-generation antimicrobial peptide dendrimer, G3KL, showed promising results regarding its antibacterial activity against MDR *A. baumannii* and *P. aeruginosa* carbapenemase producers in combination with β-lactams, aminoglycosides and ciprofloxacin (283).

Moreover, the *in vitro* synergy of 4 chimeric peptides against MDR *A. baumannii* clinical isolates in combination with cefotaxime, ciprofloxacin or erythromycin, using a checkerboard assay was studied. All four peptides exhibited significant antibacterial effects (MIC = 3.12 to 12.5 μM) against all the strains, whereas five commercial antibiotics showed little or no activity against the same pathogens (267).

Other study evaluated the *in vitro* and *in vivo* activity of the peptide IB-367 alone and in combination with imipenem, ceftazidime, piperacillin, ciprofloxacin, amikacin or colistin against different MDR bacteria (284). They found synergism, using the checkerboard titration assay, of the peptide IB-367 in combination with imipenem or colistin against *A. baumannii*, *P. aeruginosa*, *E. coli* and *K. pneumoniae*, and in the murine wound infection model in mice treated with topical IB-367 and infected with *P. aeruginosa* and *E. coli* (284).

In addition, another study evaluated the antimicrobial activity of a novel synthetic inhibitor (MD3) of type I signal peptidase in combination with outer membrane-permeabilizing agents such as sodium hexametaphosphate or colistin against a collection of *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* (261, 285). They observed that MD3 together with colistin was a more potent combination than MD3 with NaHMP. Moreover, the most dramatic antimicrobial activity was observed against both colistin-susceptible and colistin-resistant MDR *A. baumannii* strains (261).

b. Small molecules

The small molecule BAS00127538, which inhibits the lipid II, was evaluated *in vitro* against carbapenem- and/or colistin-susceptible or resistant *A. baumannii* isolates in comparison to colistin, meropenem and vancomycin, and synergy studies for BAS00127538 in combination with colistin were also assessed. This small molecule showed synergy with colistin against 84.6 % of the isolates, with no apparent association with colistin or carbapenem-resistant profiles (257).

Moreover, the *in vitro* synergistic activity of piperazine derivatives and sub-inhibitory concentrations of colistin was assessed against clinical colistin-resistant *A. baumannii* strains (286) but found synergy with one of these compounds against only 2 out of 15 strains. In another study, a 2-AI containing small molecule adjuvant showed a considerable reduction of the colistin MIC against each *A. baumannii* strain studied. Additionally, this molecule downregulates the pmrCAB operon and suppresses lipid A modification, and in the process restores colistin sensitivity to colistin resistant *A. baumannii* and *K. pneumoniae* (287).

The combination treatment of the molecule G10KHc and tobramycin showed a synergistic-like enhancement in killing activity of *P. aeruginosa* biofilms and planktonic cultures, caused by the increase in tobramycin uptake due to G10KHc-mediated cell membrane disruption (288).

In vitro interaction between natural extracts of watercress and gentamicin was carried out against *E. coli* ESBL, showing that there is an increase in antibacterial activity of the antibiotic when it is combined with the extracts (289). Moreover, another study

demonstrates that the combination of tetracycline and an essential oil of *Libanotis montana* showed synergistic or additive interactions (290).

c. Other compounds

It has been also evaluated the potential of the anthracycline Antibiotic 301A(1) to enhance the activity of clinically used antibacterial agents, showing high *in vitro* synergy with rifampin and moderate synergy with linezolid against *E. coli* ad *A. baumannii*, as an adjuvant capable of sensitizing GNB to antibiotics to which they are ordinarily intrinsically resistant (291).

Furthermore, the activity of natural products from plants has some advantages due to its chemical diversity and availability (292). For instance, plant phenolics (ellagic and tannic acids) has been studied *in vitro*, as adjuvants to enhance the activity of antibiotics against MDR *A. baumannii* strains to a large variety of antibiotics, as aminoglycosides (neomycin, amikacin, tobramycin and gentamicin), β-lactams (ampicillin and imipenem), fusidic acid, macrolides (erythromycin and azithromycin), rifampin, tetracycline and aminocoumarins (293). In this study it was demonstrated that ellagic acid improves the antibacterial activity of aminocoumarins, rifampicin and fusidic acid against *A. baumannii*, representing a promising antibiotic adjuvant lead compound (293).

Another compound such as disodium edetate (CSE-1034), a potent class B metallo-β-lactamase inhibitor, has been shown to be useful as an adjuvant antibiotic in combination with ceftriaxone plus sulbactam. CSE-1034 was active against extended spectrum β-lactamase and metallo-β-lactamase producers MDR *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* clinical isolates, concluding that this antibiotic adjuvant is effective restoring the *in vitro* activity of some β-lactams (294).

With respect to gallium nitrate [$Ga(NO_3)_3$], besides being active alone, it also shows *in vitro* synergy with colistin against both colistin-susceptible and colistin-resistant *A. baumannii* strains (278).

5.2. Other approaches

5.2.1. Vaccines

Various studies have been carried out to identify vaccine targets to provide a novel strategy for protection against MDR Gram-negative infections (295, 296). Currently, vaccines are being developed for multiple bacterial species that produce difficult-to-treat infections.

The clinical implementation of vaccination strategies for the prevention of antibiotic-resistant infections would present health and economic benefits, however, the development of vaccines for these infections presents some challenges, as targeting population selection, vaccine administration and antigen identification (297). Therefore, using vaccination as an approach for combating antibiotic-resistant infections has both benefits and limitations.

5.2.2. Phage therapy

Bacteriophages are viruses that infect and kill bacteria. Thus bacteriophage therapy is one of the emerging methods used to overcome bacterial infections (298, 299). The use of phages is supposed to be advantageous over antibiotics, because bacteriophages are highly specific and have no effect against animal cells, and in addition, their isolation is a relatively rapid process. It is of interest the applications of bacteriophages as potentially powerful antibacterial agents, due to the emergence of drug-resistant pathogens and the lack of optimal antimicrobials (300).

5.2.3. Iron chelation

Siderophores are very important compounds for the survival of bacteria. Pathogen iron acquisition could be further disrupted by using biologically compatible chelators (301) or by introducing gallium as a competitor (302). Therefore, inhibition of their activity is an alternative for antibiotics or may even be a complement of treatment with antibiotics.

5.2.4. Photodynamic therapy

Photodynamic therapy was developed a long time ago, but it was used for other purposes such as cancer or ophthalmology, and for disinfection of clinical products (303). Antimicrobial photodynamic therapy is starting to be considered as a promising alternative approach to resistant infections and has the further advantage of not leading to the selection of resistant strains (304). It appears to be suit for the treatment of superficial skin and soft tissue infections (305).

II. FUNDAMENTS

Fundaments

The emergence of infections by MDR GNB is a well-recognized global health threat in urgent need of effective solutions. Due to the high rate of resistant strains to all or almost all the antimicrobials used in the clinical practice, these pathogens cause difficult-to-treat infections associated to important mortality rates. Furthermore, this problem is aggravated by the lack of development of other therapeutic options to treat those infections by GNB. For this reason, there is an urgent need to address the challenge of developing new drugs or strategies to combat MDR and PDR resistant GNB, which cause severe infections in humans. The development of non-antimicrobial therapeutic alternatives is among the most promising approaches.

Single-pathogen therapies that block bacterial virulence factors without inhibiting bacterial growth emerge as new encouraging strategy, since they present less selective pressure for the generation of resistance. Many bacterial pathogens interact with host environment using their OMPs, as OmpA or its homologous protein, in order to induce the expression of virulence factors, to invade tissues, and to escape the immune system. Given the major role of OmpA in promoting bacteria Gram-negative virulence, we have designed and tested the effectiveness of OmpA inhibitors *in vitro* and *in vivo* in preventing infection by GNB such as *A. baumannii*, *P. aeruginosa* and *E. coli*.

Another therapeutic alternative adjuvant to antimicrobial treatment is to stimulate the immune system to block the progression of the infection. Sepsis and pneumonia are characterized by the induction of the inflammatory cascade that is lesional for different organs when is disproportionate. In hospitalized patients, *A. baumannii* and *P. aeruginosa* are among the most frequent etiologies. Preliminary studies of our group have shown that the use of LPC, an immunomodulator chemoattractant factor, as a preventive treatment increases the mice survival to 66.67 % after infection with *A. baumannii*. However, no studies have evaluated its use in combination therapy with other antibiotics. Therefore, we have evaluated the therapeutic potential of LPC in the combined treatment against *A. baumannii* and *P. aeruginosa* in murine models of peritoneal sepsis and pneumonia.

III. HYPOTHESES

Hypotheses

The hypotheses of this doctoral thesis are:

Chapter I

1. OmpA inhibitors reduce the adherence and invasion of *A. baumannii*, *P. aeruginosa* and *E. coli* in human lung epithelial cells and their cell death.

2. The use of OmpA inhibitors in monotherapy and in combination with colistin improves the prognosis of infection by *A. baumannii*, *P. aeruginosa* and *E. coli* in murine experimental models of peritoneal sepsis.

Chapter II

3. Combined treatment of LPC with colistin, imipenem or tigecycline improves the evolution of infection caused by *A. baumannii* in murine experimental models of peritoneal sepsis and pneumonia.

4. Combined treatment of LPC with imipenem or ceftazidime improves the evolution of infection caused by *P. aeruginosa* in murine experimental models of peritoneal sepsis and pneumonia.

IV. OBJECTIVES

Objectives

The general aim of this Doctoral Thesis is to contribute to the development and evaluation of non-antimicrobial strategies for the prevention and treatment of infections caused by GNB. The specific objectives of each chapter are the following:

Objectives of chapter I

1. To design and produce OmpA inhibitors.

2. To characterize the effect of OmpA inhibitors on the interaction between *A. baumannii*, *P. aeruginosa* and *E. coli* and human lung epithelial cells (A549).

3. To evaluate the therapeutic efficacy of OmpA inhibitors against *A. baumannii*, *P. aeruginosa* and *E. coli* in a murine experimental model of peritoneal sepsis.

4. To study the *in vitro* activity of OmpA inhibitor in combination with colistin against colistin susceptible and resistant *A. baumannii*.

5. To evaluate the therapeutic efficacy of the OmpA inhibitor in combination with colistin in a murine peritoneal sepsis model by colistin susceptible and resistant *A. baumannii*.

Objectives of chapter II

1. To evaluate the efficacy of LPC *in vivo* in combination with imipenem, tigecycline or colistin in murine experimental model of peritoneal sepsis caused by susceptible and MDR *A. baumannii*.

2. To assess the efficacy of LPC *in vivo* in combination with imipenem, tigecycline or colistin in murine experimental model of pneumonia caused by MDR *A. baumannii*.

3. To evaluate the efficacy of LPC *in vivo* in combination with imipenem or ceftazidime in murine experimental model of peritoneal sepsis caused by susceptible and MDR *P. aeruginosa*.

Objectives

4. To assess the efficacy of LPC *in vivo* in combination with imipenem or ceftazidime in murine experimental model of pneumonia caused by susceptible and MDR *P. aeruginosa*.

5. To study the release of pro- and anti-inflammatory cytokines induced by *P. aeruginosa* in murine peritoneal sepsis and pneumonia experimental models.

V. RESULTS

1. Chapter I

Chapter I. Article I

1.1. Article I. Impact of OmpA inhibition on Gram-negative bacilli virulence and antimicrobial resistance

OmpA and its homologues are a main virulence factor for GNB such as *A. baumannii*, *P. aeruginosa* and *E. coli*. OmpA is involved in the adherence of these GNB to biotic and abiotic surfaces and in the host death *in vitro* and *in vivo*. Therefore, OmpA is a good candidate for peptides development to treat infections caused by these GNB. Here, we aimed to evaluate the *in vitro* and *in vivo* activity of an OmpA inhibitor against GNB infections, and the activity of its combination with colistin.

One negative control and six positive OmpA inhibitors were designed and synthesized *in silico* in collaboration with Dr. Ernest Giralt team, and AOA-2, the best candidate, was chosen for the *in vitro* and *in vivo* studies. The *in vitro* effect on interaction of AOA-2 between *A. baumannii* ATCC 17978, *P. aeruginosa* Pa01, *E. coli* ATCC 25922 strains, and human lung epithelial cells (A549) was characterized by adherence, immunofluorescence, fibronectin binding, and cell viability assays. Moreover, AOA-2 effect on biofilm formation by standard and clinical strains of *A. baumannii* (n=10), *P. aeruginosa* (n=9), and *E. coli* (n=7) was determined. In a murine peritoneal sepsis model, the therapeutic efficacy of AOA-2 (10 mg/kg/day, intraperitoneally) against these pathogens was evaluated and the bacterial load (spleen and lungs), and bacteremia and mice survival were analyzed. In addition, MICs for colistin against *A. baumannii* colistin-susceptible and colistin-resistant strains in presence and absence of AOA-2 were determined. The synergy between AOA-2 and colistin was evaluated using time-kill curves, and the protein profile in the presence and absence of AOA-2 was analyzed by SDS-PAGE. A *knockout* strain deficient in the *omp25* gene and its complemented strain were constructed, and the same experiments were carried out. Finally, the *in vivo* therapeutic efficacy of colistin alone or in combination with AOA-2 was assessed in a murine experimental model of peritoneal sepsis by *A. baumannii* strain ATCC 17978.

A significant reduction of bacterial adherence to A549 cells and binding to fibronectin was observed after incubating ATCC 17978, Pa01 and ATCC 25922 strains with 0.25 and 0.5 mg/mL AOA-2, as well as protection of A549 cells from death caused by ATCC 17978 and Pa01 strains. AOA-2 reduced significantly the biofilm formation by *A. baumannii*, *P. aeruginosa*, and *E. coli* strains. Moreover, in the peritoneal sepsis model, AOA-2 treatment reduced the bacterial load for ATCC 17978 strain in spleen

and lungs by difference of 4.54 and 4.34 log CFU/g, respectively, and the positive blood culture by 70 %, and increased the mice survival by 70 %. For Pa01 strain, AOA-2 treatment reduced the bacterial load in spleen and lungs by difference of 4.04 log and 4.29 log CFU/g, respectively, and the positive blood culture by 40 %, and increased the mice survival by 40%. For *E. coli* ATCC 25922 strain, AOA-2 treatment reduced the bacterial load in spleen and lungs by difference of 3.36 and 3.47 log CFU/g, respectively, and the positive blood culture by 40 %, and increased the mice survival by 40 %.

With respect to the combination of AOA-2 with colistin, a significant reduction was observed in MICs of colistin in the presence of AOA-2 for colistin-susceptible and colistin-resistant strains, time-kill curves showed synergistic activity between AOA-2 and colistin and the profile of OMPs in each strain treated with AOA-2 exhibited an overexpression of the Omp25 protein. In the murine peritoneal sepsis model, the treatment with colistin in combination with AOA-2 against ATCC 17978 strain reduced bacterial concentration in spleen and lungs and bacteremia and increased mice mortality and with respect to monotherapy with colistin.

These data indicate that the inhibition of OmpA by AOA-2 could protect against infections by GNB, and that there is a synergistic activity between AOA-2 and colistin *in vitro*, which is effective reducing the infection by *A. baumannii* in peritoneal sepsis experimental model.

Chapter I. Article I

Title: Impact of OmpA inhibition on Gram-negative bacilli virulence and antimicrobial resistance

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Author contributions

M.T., J.V., J.P., E.G., Y.S. conceived the study and designed the experiments. X.V.F., R.P.M, V.S.E., M.V, R.A.A, N.B., M.E.P.I., M.K. performed experiments and interpreted data. J.P. and Y.S. wrote the manuscript with the input of all the other authors.

Competing financial interests

No conflict of interests to declare

Chapter I. Article I

Abstract

Preventing the adhesion of pathogens to host cells provides an additional approach to tackling multidrug-resistant bacteria. In this regard, the identification of outer membrane protein A (OmpA) as a key bacterial virulence factor has been a major breakthrough. Using virtual screening based on knowledge of the target, we identified a cyclic hexapeptide AOA-2 that inhibits the adhesion of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Escherichia coli* to host cells, thereby preventing the development of infection *in vitro* and *in vivo*. AOA-2 does not inhibit bacterial growth; however, it potentiates the effectiveness of colistin *in vitro* and *in vivo*. Inhibition of OmpA offers a strategy to address the urgent need for treatments for infections caused by Gram-negative bacilli.

Main Text

Nowadays, the treatment of infections, especially bacterial strains resistant to all the antibiotics, is a major concern. The number of antibiotics approved by the FDA cannot reach the pace which resistance is acquired by bacteria, and therefore there is an urgent need to find new antibiotics against extended-(XDR) and pandrug-(PDR) resistant Gram-negative bacilli (GNB)[1]. So far, most of the antibiotics used are either bactericidal or bacteriostatic and most of them used against a broad spectrum of bacteria. To circumvent the problem of antibiotics resistance there are two key points that could improve the current situation. Single-pathogen therapies improve the cost-effective therapy[2], and tackling bacterial virulence factors without inhibiting bacterial growth, since they present less selective pressure for the generation of resistance[3,4]. Many bacterial pathogens use their outer membrane proteins (OMPs) to interact with host environment in order to induce the expression of virulence factors, to invade tissues, and to escape the immune system[5-7]. These pathogens appear to use the outer membrane protein A (OmpA), among others, to contact host cells and to mediate bacterial entry[8]. OmpA is a beta-barrel porin that is highly conserved among bacterial species, especially throughout GNB[7]. This protein is multifunctional, with a variety of *in vitro* and *in vivo* biological properties of interest. It has been shown to be involved in adherence to epithelial cells[9-11], translocation into epithelial cells nucleus[12], induction of epithelial cell death[12-14], biofilm formation[10,15], and binding to factor H - the latter postulated to allow bacteria to develop serum-resistance[14,16]. Given the major role of

OmpA in promoting the GNB virulence, here we designed and tested the effectiveness of OmpA inhibitors to prevent infection by GNB.

Cyclic peptides are really promising drugs due to its high half-life time compared to linear peptides, some peptides are in clinical trials[17]. The size of the cyclic peptides was chosen according to the size of target. A library of 26 cyclic hexapeptides was screened computationally to identify OmpA inhibitors, these 26 cyclic peptides have two common features, all peptides are symmetric and have two fixed proline (to ease the cyclization). As no crystal structure of *A. baumannii* OmpA was found in the Protein Data Bank, a homology model using i-TASSER server was built using the *A. baumannii* OmpA sequence, and the crystallized structure of *Escherichia coli* as a template. Surprinsigly, all the peptides containing tryptophan and arginine (in addition to proline present in all the peptides) showed better *in silico* activity compared with other hexapeptides (Table S2). Four of these peptides with high affinity *in silico* were synthesized, together with two other peptides with important changes in the amino acid properties in order to validate the *in silico* assay. In addition, the lineal version of the most active peptide *in silico* was synthesized, and was used as negative control (Fig. S1, Table S3).

In an initial screening of the synthesized peptides their bactericidal activity and toxicity in human lung epithelial cells (A549) were evaluated, together with their capacity to reduce bacterial adherence to A549 cells. One compound, AOA-2 (&Trp-D-Pro-Arg-Trp-D-Pro-Arg&) (Fig. 1A), was selected on the basis of its greater capacity to reduce adherence than other hexapeptides (Fig. S2) and its minimal toxicity in this cell line (Table S4).

Previously, we reported that *OmpA*-deficient *A. baumannii* is less adherent to biotic and abiotic surfaces[18] and that recombinant OmpA shows high binding to fibronectin, an extracellular matrix protein[18]. *Pseudomonas aeruginosa* and *E. coli* encode OmpA homologues (58% similarity)[19], which also mediate the pathogenic roles of these bacteria[9]. We found that 0.25 mg/mL AOA-2 reduced the *in vitro* adherence of *A. baumannii* to cells more than 60%, and it also reduced significantly the adherence of *P. aeruginosa*, and *E. coli* strains to A549 cells (Fig. 1C and Fig. 1D), in particular to fibronectin (Fig. 1E). Unlike conventional antimicrobial drugs, AOA-2 has no bacteriostatic or bactericidal activity against the above mentioned strains (Fig. S4). Interestingly, AOA-2 also diminished the biofilm formation, a structure involved in the persistence of bacterial infections[20], by standard and clinical isolates of *A. baumannii*, *P. aeruginosa*, and *E. coli* (Table S5).

Consequently, cell death dependence on bacterial adherence[11,21] was prevented in the presence of 0.25 and 0.5 mg/mL AOA-2, except for ATCC 25922 (Fig. 1F), due to the effect of the cyclic peptide. The failure of AOA-2 to prevent cell death by *E. coli* may be attributable to the presence of other highly virulent factors circumventing the loss of OmpA[22].

Studies with AOA-2 in mice did not reveal any evidence of toxicity at a dose ≤ 40 mg/kg (Table S6). Therefore, in a peritoneal sepsis murine model, administration of 10 mg/kg AOA-2 to mice 2 hours after intraperitoneal (i.p.) challenge with minimal lethal doses of ATCC 17978, PaO1, and ATCC 25922 strains reduced the bacterial load of these bacteria in spleen (4.54, 4.04, and 3.36 log CFU/g, respectively) and lungs (4.34, 4.29, and 3.47 log CFU/g, respectively) (Fig. 1G). Also, positive blood cultures and mouse mortality were also decreased (Fig. 1H). Pharmacokinetic studies indicated that AOA-2 showed good bioavailability when administered i.p. to mice (Fig. S5).

So far we have described AOA-2, a highly effective cyclic peptide against *A. baumannii* that acts by blocking the interaction between bacteria and host-cell, however the activity of this compound will improve if it could be co-administered with other antimicrobial agents. Therapies combining antimicrobial agents and peptides are among the new promising strategies to treat bacterial infections[23]. *OmpA* disruption leads to decreased minimal inhibitory concentrations (MICs) of chloramphenicol, aztreonam, nalidixic acid, and polymyxin against *A. baumannii*[24,25]. Here we sought to examine the capacity of AOA-2 to potentiate the activity of these antimicrobial agents on *A. baumannii* clinical isolates. Firstly, MIC data show that AOA-2 increased the susceptibility of ATCC 17978 to chloramphenicol, aztreonam, nalidixic acid, and colistin, in agreement with previous observations[24,25] (Table S7). Indeed, addition of AOA-2 to laboratory and to colistin-susceptible (Col-S, n=6), and colistin-resistant (Col-R, n=16) clinical isolates of *A. baumannii* strains increased their susceptibility to colistin; thus, the MIC_{50} for colistin decreased from 32 to 2 µg/mL in the presence of AOA-2 (Table S8). Next, we examined the capacity of AOA-2 in combination with colistin to kill Col-S ATCC 17978 and Col-R #11 *A. baumannii* strains in a time-kill assay. In this regard, 125 µg/mL AOA-2 in combination with a colistin sub-MIC (0.25 µg/mL) showed a greater bactericidal capacity than colistin alone, decreasing the bacterial cell count by 5.54 log CFU/mL after 24 h. The combination of 12.5 µg/mL AOA-2, equivalent to its C_{max} in mice treated with 10 mg/kg AOA-2 (Fig. S5), with 0.25 µg/mL colistin reduced bacterial concentration by 5.41 log CFU/mL with respect to colistin

alone after 24 h (Fig. 2A). Furthermore, the combination of 125 µg/mL AOA-2 with 8 µg/mL colistin, colistin sub-MIC for the #11 strain, showed excellent bactericidal activity against the #11 strain, decreasing the bacterial cell counts with respect to colistin by 3.22 and 2.16 log CFU/mL after 2 and 4 h, respectively. However, bacterial regrowth occurred after 4 h; in addition, the combination of 12.5 µg/mL AOA-2 with 8 µg/mL colistin showed limited bactericidal activity (Fig. 2A). In a control experiment, AOA-2, SXV4 (negative control lineal peptide), or the combinations of SXV4 with 0.25 or 8 µg/mL colistin, showed no bactericidal activity against ATCC 17978 and #11 strains, respectively (Fig. 2A). Since colistin half-life in bacterial culture broth is 4 h[26,27] and AOA-2 was stable in human serum during 24 h (data not shown), we cannot rule out the possibility that the observed bacterial regrowth of the #11 strain in the presence of AOA-2 and 8 µg/mL colistin (Fig. 2A) is due to colistin degradation in the bacterial culture broth. Consequently, to maintain the colistin concentration, in the following experiments we added 4 µg/mL of colistin 4 h post-incubation with AOA-2 and 8 µg/mL colistin. This approach produced greater activity and synergy between AOA-2 and colistin (Fig. 2B). In the control experiment, addition of a second dose of SXV4 or AOA-2, 4 h post-incubation with SXV4 or AOA-2 and 8 µg/mL colistin, did not significantly affect the regrowth of the #11 strain (Fig. 2B).

Because AOA-2 showed synergy with colistin against Col-S and Col-R *A. baumannii* strains, we suggest that AOA-2 may regulate the OMPs expression, as previously reported in other study model[28]. AOA-2 increased the expression of Omp25, identified by MALDI-TOF-TOF, in the ATCC 17978 and in the #11 strain and, in parallel, conferred a ≥ 33- and 8-fold reduction of colistin MIC, respectively (Fig. 2C). The *omp25* gene transcription in 16 clinical Col-R isolates was notably reduced compared to ATCC 17978 and 11 clinical Col-S isolates. Importantly, the incubation of the 16 Col-R strains with AOA-2 increased *omp25* transcription (Fig. 2D). Moreover, the generation of an *omp25*-deficient mutant (Δ*omp25*) from the ATCC 17978 strain increased its colistin MIC from 0.5 to 2 µg/mL. The complementation of Δ*omp25* strain restored the wild-type phenotype. AOA-2 did not significantly reduce the colistin MIC of the Δ*omp25* strain (only 2-folds), unlike the complemented *omp25* mutant, for which the colistin MIC was reduced 8.33-fold (Fig. 2E).

Additional experiments were performed to confirm the role of Omp25 in the synergy between AOA-2 and colistin. AOA-2 in the presence of 0.75 µg/mL colistin, a colistin sub-MIC for the

Δ*omp25* strain, showed lower bactericidal activity against the *omp25* mutant strain than that of the wild-type strain, decreasing the bacterial cell count after 24 h to 1.73 log CFU/mL *vs*. 5.54 log CFU/mL, for Δ*omp25* and ATCC 17978 strains, respectively. Interestingly, the combination of 125 µg/mL AOA-2 with 0.125 µg/mL colistin, a colistin sub-MIC for the complemented *omp25* mutant strain, almost completely restored the activity of AOA-2 on the wild-type strain (Fig. 2F).

In a murine model of peritoneal sepsis, administration of 10 mg/kg AOA-2 in combination with a sub-optimal dose of colistin (10 mg/kg/d) (Fig. 3A, Table S9) to mice after i.p. administration of a minimal lethal dose of ATCC 17978 or clinical isolate #11 reduced the bacterial load of both strains in spleen (2.75 and 2.13 log CFU/g, respectively) and in lungs (3.22 and 2.33 log CFU/g, respectively) compared with controls (Fig. 3B). In contrast, colistin alone decreased bacterial spleen and lung loads of the ATCC 17978 and clinical #11 strains in 0.04 and 0.19 log CFU/g and 0.27 and 0.32 log CFU/g, respectively, respect to the controls. Survival and absence of bacteremia showed a greater increase in mice receiving combined treatment of AOA-2 and colistin than in those receiving colistin alone or in controls (Fig. 3B).

A number of infections caused by MDR strains require the use of colistin, but these bacteria rapidly acquire specific resistance mechanisms against this drug[29]. The rate of colistin resistance currently varies between 3 and 28% worldwide[30]. In this context, the development of this non-antimicrobial therapeutic approach, which can, in combination, improve the antimicrobial activity of the scarce and non-optimal antimicrobial agents clinically available, will potentially cover the urgent need for treating infections caused by XDR and PDR microorganisms.

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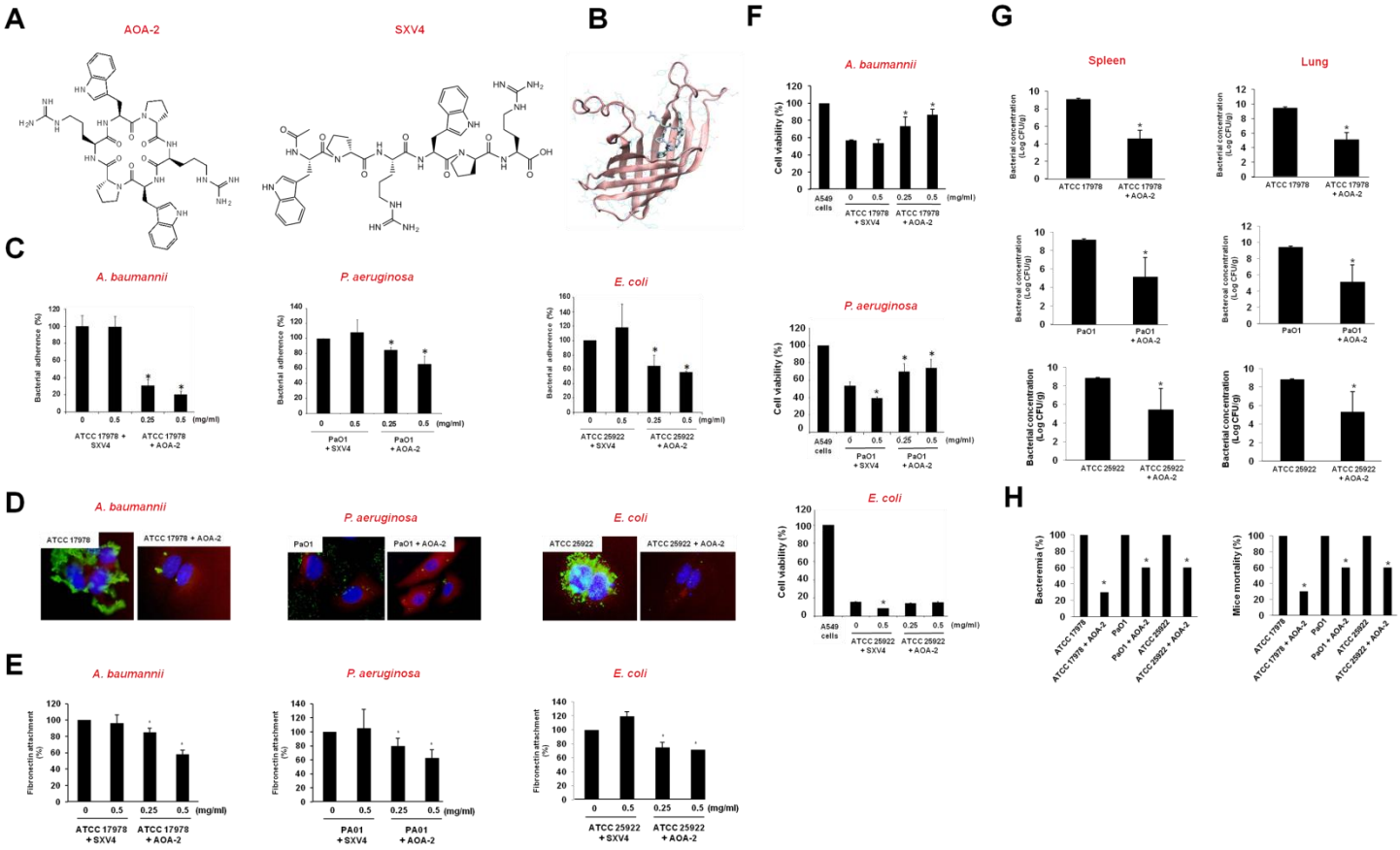

Fig. 1. AOA-2 inhibits *A. baumannii*, *P. aeruginosa* and *E. coli* virulence *in vitro* and *in vivo*. (**A**) Chemical structure of AOA-2 and SXV4 (negative control lineal peptide). (**B**) Three-dimensional representation of AOA-2 and OmpA complex. (**C**) Adhesion of ATCC 17978, PaO1, and ATCC 25922 strains to A549 cells. A549 cells were infected with 10^8 CFU/mL of each strain for 2 h in the presence of AOA-2 or SXV4. (**D**) Immunostaining of fibronectin of A549 cells (red) and ATCC 17978, PaO1, and ATCC 25922 strains (green) pretreated with AOA-2, after bacterial adherence for 2 h, was performed by specific primary antibodies against these strains and their respective secondary antibodies. Blue staining shows the location of A549 cell nuclei. (**E**) Bacterial interaction with immobilized fibronectin. Each strain pretreated with AOA-2 or SXV4 were incubated in a fibronectin-coated 96-well plate for 2 h. **(F)** Cell death induced by ATCC 17978, PaO1, and ATCC 25922 strains. A549 cells were infected for 24 h with 10^8 CFU/mL of each strain pretreated with AOA-2 or SXV4. Bacterial cytotoxicity was assessed using the MTT assay. Representative results of 3 independent experiments are shown; data are means ± SEM. (**G**) CFU/g of ATCC 17978, PaO1, or ATCC 25922 strains harvested from spleen and lungs of mice infected intraperitoneally with the MLD of each strain and treated or not with AOA-2 (10 mg/kg/d, for 3 days) 2 h after bacterial inoculation. (**H**) Bacteremia and survival from the previous mouse groups. *P<0.05: between untreated and treated groups.

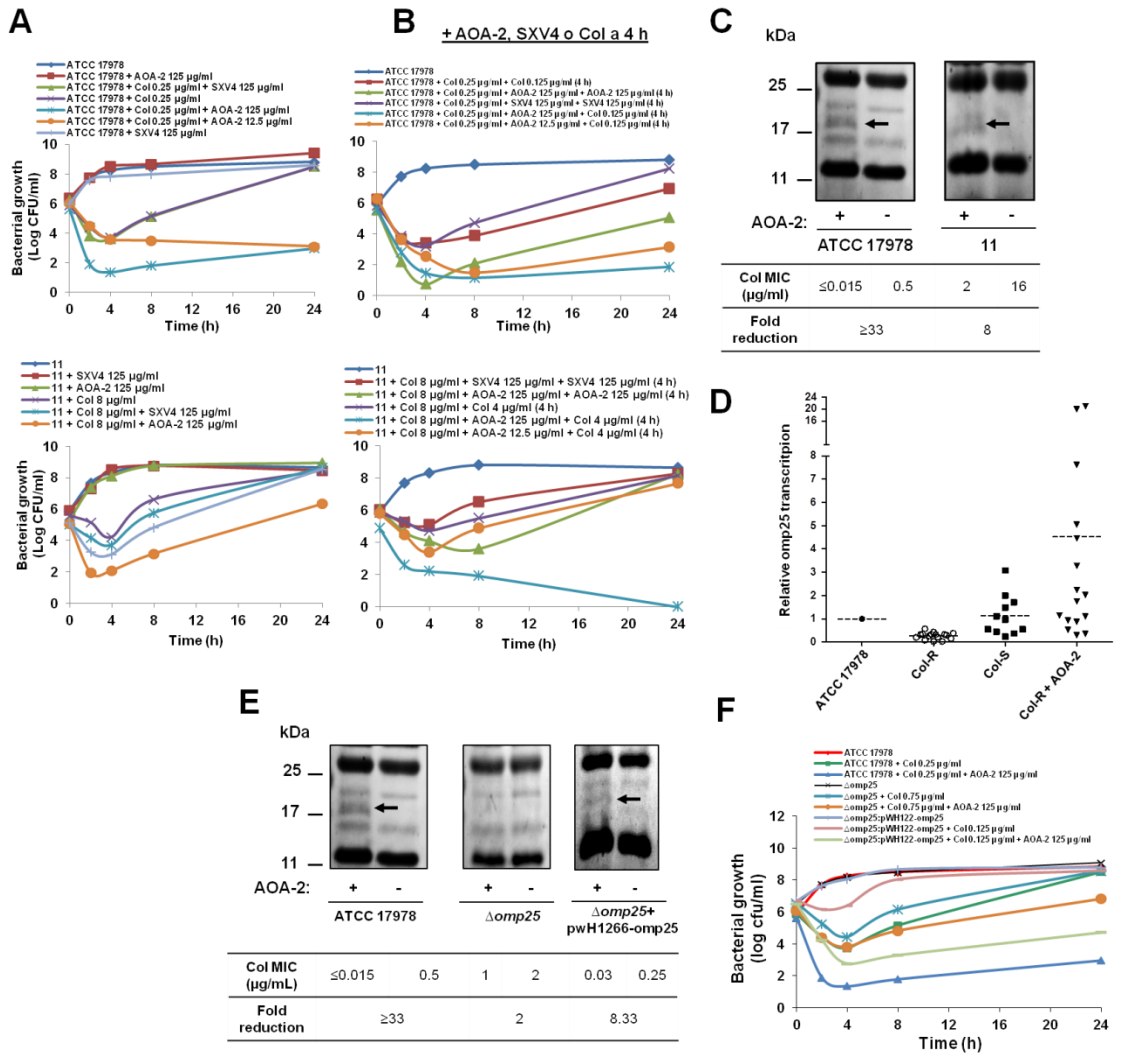

Fig. 2. AOA-2 potentiates the colistin activity against *A. baumannii in vitro*. (**A**) Time-kill curves of *A. baumannii* ATCC 17978 and #11 clinical strains in the presence of AOA-2 or SXV4 (0, 12.5 or 125 µg/mL), colistin (sub-MIC) alone or in combination with AOA-2, SXV4 or colistin for 24 h. (**B**) Time-kill curves of the ATCC 17978 and #11 clinical strains in the presence of different combinations between AOA-2, SXV4 or colistin, and AOA-2, SXV4 or colistin added for second time 4 h after bacterial addition, for 24 h. (**C**) MICs of colistin with or without 125 µg/mL AOA-2 of ATCC 17978 and #11 clinical strains, and SDS-PAGE of their OMPs with or without 125 µg/mL AOA-2. (**D**) *omp25* transcription (qRT-PCR) in *A. baumannii* ATCC 17978 strain, 11 Col-S and 16 Col-R clinical isolates, and 16 Col-R clinical isolates with 125 µg/mL AOA-2. (**E**) MICs of colistin in the presence or absences of 125 µg/mL AOA-2 on ATCC 17978, Δ*omp25* and Δ*omp25*+pwH1266-*omp25* strains, and SDS-PAGE of their OMPs with or without 125 µg/mL AOA-2. **(F)** Time-kill curves of ATCC 17978, Δ*omp25*, and Δ*omp25*+pwH1266-*omp25* strains in presence of AOA-2 (125 µg/mL), colistin alone (sub-MIC), or in combination for 24 h.

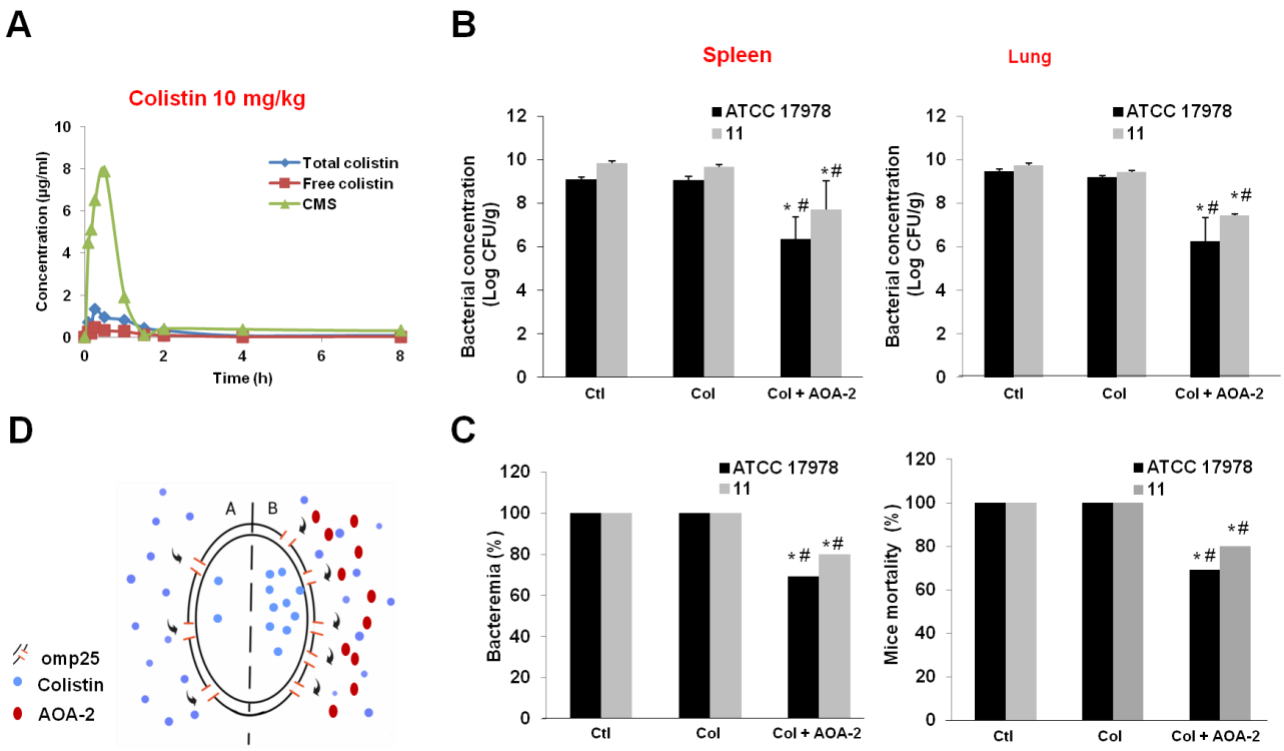

Fig. 3. AOA-2 potentiates colistin activity against *A. baumannii in vivo*. (**A**) Serum CMS and total and free colistin pharmacokinetics. Colistin was administered to mice at a dose of 10 mg/kg, and the serum CMS and total and free colistin concentrations were analyzed for 8 h. (**B**) CFU/g of ATCC 17978 or #11 strains harvested from spleen and lungs of mice infected intraperitoneally with MLD of ATCC 17978 or #11 clinical strains, from controls without treatment, colistin (10 mg/kg/d, during 3 days) or colistin in combination with AOA-2 (10 mg/kg/d, during 3 days) treated groups. (**C**) Bacteremia and mortality after intraperitoneal infection with MLD of ATCC 17978 or #11 strains of controls and of mice treated with colistin (10 mg/kg/d, during 3 days) or colistin (10 mg/kg/d, for 3 days) in combination with AOA-2 (10 mg/kg/d, for 3 days). (**D**) Proposed scheme for the mechanism of AOA-2 action in the presence of colistin. We propose that AOA-2 increases the expression of Omp25 in *A. baumannii* and allows greater entrance of colistin into *A. baumannii* cells. **P*<0.05: between colistin+AOA-2 and colistin. $^{\#}$*P*<0.05: between colistin+AOA-2 and control. Ctl: control, Col: colistin.

Chapter I. Article I: supplementary material

Supplementary Materials

Materials and Methods

Bacterial strains

The list of bacterial strains was regrouped in table S1.

Docking and molecular modeling

Glide XP scoring function and molecular docking software from SCHRODINGER package (¨http://www.schrodinger.com¨) were used. Protein structure was obtained with the help of homology modeling technique. Homology modeling constructs a model of the "target" protein from its amino acid sequence and an experimental three dimensional structure (X-Ray crystal or NMR) of a related homologous protein (the "template"). I-TASSER online server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used for homology modeling. The amino acid sequence was taken from Genbank, and template structures used in the modeling were taken from the ompA structures present in the PDB. Best ranked model was prepared (all preparation was done with SCHRODINGER including short MD simulations) for docking calculations. A virtual library of cyclic hexapeptides was used for docking.

Materials for peptide synthesis

Protected amino acids and resins were supplied by Luxembourg Industries (Tel-Aviv, Israel), Neosystem (Strasbourg, France), Calbiochem-Novabiochem AG (Laüfelfingen, Switzerland), PolyPeptide Laboratories (Torrance, CA USA), Bachem AG (Bubendorf, Switzerland), and Iris Biotech (Marktredwitz, Germany). PyBOP was provided by Calbiochem-Novabiochem AG.Piperidine, was from SDS (Peypin, France); *N,N*-diisopropylethylamine (DIEA) was obtained from Merck (Darmstadt, Germany) and Triisopropylsilane (TIS) andninhydrin were from FlukaChemika (Buchs, Switzerland). HOAt was purchased from GL Biochem Shanghai Ltd. (Shanghai, China). Solvents for peptide synthesis and RP-HPLC (dimethylformamide (DMF), dichloromethane (DCM) and acetonitrile (MeCN)) were from Scharlau or SDS (Barcelona, Spain). Trifluoroacetic acid (TFA) was purchased from KaliChemie (Bad Wimpfen,

Germany). The other chemicals used were from Aldrich (Milwaukee, WI USA) and were of the highest purity commercially available.

Peptide synthesis

Peptides were synthesized on a 2-Chlorotrytil chloride resin by solid-phase peptide synthesis using the 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/*t*Bu) strategy. N^{α}-Fmoc-protected amino acids (3 eq)/HOAt (3 eq), PyBOP (4 eq), and DIEA (6 eq) were used for couplings. The Fmoc protecting group was removed by treatment with a solution of 20% piperidine in DMF. Peptides were cleaved using 2% TFA in DCM. For the cyclization step, the solvent used was DCM/DMF (98:2), PyAOP (2 eq) was dissolved in DMF and the peptide (5 mM) in DCM and once is mixed in the right proportions, 6 eq of DIEA were added, the reaction was completed in 2 or 3 h. After the cyclization was carried out, the deprotection of the side chains was performed using TFA/TIS/ H_2O (95:2.5:2.5). The peptides were analysed at λ = 220 nm by analytical HPLC [Waters Alliance 2695 separation module equipped with a 2998 photodiode array detector, Sunfire C_{18} column (100 mm x 4.6 mm x 3.5 mm, 100 Å, Waters), and Empower software; flow rate = 1 mL/min. The peptides were then purified by semi-preparative HPLC [Waters 2700 Sample Manager equipped with a Waters 2487 dual λ absorbance detector, a Waters 600 controller, a Waters fraction collection II, a Symmetry C_{18} column (100 mm x 30 mm, 5 mm, 100 Å, Waters) and Millenium chromatography manager software]. Flow rate = 15 mL/min; solvents: A = 0.1% trifluoroacetic acid in water, and B = 0.05% trifluoroacetic acid in acetonitrile. Peptides were characterized by MALDI-TOF mass spectrometry (Voyager-DE RP MALDI-TOF, PE Biosystems with a N2-laser of 337 nm) and a high resolution ESI-MS model (LTQ-FT Ultra, Thermo Scientific).

Human cell culture and infection

Type II pneumocyte cell line A549 derived from a human lung carcinoma were grown in DMEM medium supplemented with 10% heat-inactivated FBS, vancomycin (50 µg/mL), gentamicin (20 µg/mL), amphotericin B (0.25 µg/mL) (Invitrogen, Spain) and 1% HEPES in a humidified incubator, 5% CO_2 at 37 ºC. A549 cells were routinely passaged every 3-4 days. The cells were

seeded 24 h in 96-well plates for cellular viability assay, and in 24 well plates for adhesion and immnunofluorescence assays.

Cellular viability

Peptide toxicity. A549 cells were incubated with AOA-1, AOA-2, AOA-3, AOA-4, AOA-5, AOA-6, or negative control peptide SXV4 (0, 0.25, 0.5 and 1 mg/mL) 48 h with 5% CO_2 at 37 °C. Prior the evaluation of the peptides cytotoxicity, A549 cells were washed three times with prewarmed PBS. Then, peptides cytotoxicity was initially assessed quantitatively by monitoring the mitochondrial reduction activity using the MTT assay as described previously[1]. The percentage of cytotoxicity was calculated from the optical density (OD) as follow: [(OD of treated cells / mean OD of non-treated cell) x 100].

Bacterial cytotoxicity. A549 cells were infected with 10^8cfu/mL of *A. baumannii*ATCC 17978, *P. aeruginosa* PaO1, and *E. coli* ATCC 25922 strains pretreated with AOA-2 or SXV4 (0, 0.25 and 0.5 mg/mL, 30 min)for 24 h with 5% CO_2 at 37 °C. Prior the evaluation of bacterial cytotoxicity, we firstly removed viable bacteria from A549 cells cultures and washed A549 cells five times with pre-warmed PBS. Then, cellular viability was assessed as indicated above.

Adhesion assays

A. baumannii ATCC 17978, *P. aeruginosa* PaO1, and *E. coli* ATCC 25922 strains were pretreated with AOA-1, AOA-2, AOA-3, AOA-4, AOA-5, AOA-6, or SXV4 (0, 0.25, 0.5 and 1 mg/mL, 30 min), and added to A549 cells for 2 h at 5% CO_2 and 37 °C. Subsequently, infected A549 cells were washed five times with pre-warmed PBS and lysed with 0.5 % Triton X-100. Diluted lysates were plated onto blood agar (Blood-Agar Columbia, Becton Dickinson Microbiology Systems, USA) and incubated at 37 °C for 24 h for enumeration of developed colonies and then the determination of the number of bacteria that attached to A549 cells.

Immunofluorescence

The A549 cells plated on coverslips were incubated with *A. baumannii* ATCC 17978, *P. aeruginosa* PaO1, and *E. coli* ATCC 25922 strains for 2 h, pre-treated with AOA-2 or SXV4 (0 and 0.25 mg/mL, 30 min) at 5% CO_2 and 37 °C. Bacterial cells were removed and A549 cells

were washed five times with cold PBS. A549 cells on the coverslips were fixed in methanol for 8 min at -20 ºC, permeabilized with 0.5% Triton X-100 and blocked with 20% pork serum in PBS. Primary antibodies: anti-OMPs of *A. baumannii* produced in mouse kindly gift by MJ McConnell, mouse anti-*P. aeruginosa* (abcam, Spain), mouse anti-*E. coli* (abcam, Spain), and rabbit anti-human fibronectin (Sigma, Spain) were used at dilution of 1:50, 1:100, 1:100 and 1:400, respectively, in PBS containing 1% BSA for 2 h. After washing with PBS, the coverslips were incubated with their respective secondary antibodies: Alexa488-conjugated goat anti-mouse IgG, and Alexa594-conjugated goat anti-rabbit IgG (Invitrogen, Spain) at dilution of 1:100, 1:200, 1:200 and 1:800, respectively, in PBS containing 1% BSA for 1 h. The fixed coverslips were incubated for 10 min at room temperature with DAPI (Applichem, Germany) (0.5 µg/mL), washed with PBS, mounted in fluorescence mounting medium (DakoCytomation, Spain), and visualized using a Leica fluorescence microscope (DM-6000; Leica Microsystems Wetzlar GmbH, Germany).

Fibronectin-binding assays

Fibronectin-binding assays were performed as described previously[2]. Briefly, *A. baumannii* ATCC 17978, *P. aeruginosa* PaO1, and *E. coli* ATCC 25922 grown overnight at 37°C in LB were resuspended in PBS and collected by centrifugation at 5,000× g for 10 min. Bacteria were washed twice in sterile PBS, resuspended in the same sterile buffer, and incubated with AOA-2 or SXV4 (0, 0.25 and 0.5 mg/mL, 30 min). Then, 50 µL of bacterial suspension were mixed with 50 µL of PBS, added to fibronectin coated wells and incubated 2 h at room temperature for bacterial adsorption. Non-adhered bacteria were discarded and wells were washed six times with sterile PBS to remove unbound bacteria. Adherent bacteria were then collected with 125 µL of sterile PBS containing 0.5% Triton X-100. Diluted lysates were plated onto sheep blood agar and incubated at 37°C for 24 h for enumeration of developed colonies and then the determination of the number of bacteria that attached to fibronectin.

***In vitro* susceptibility testing and time-kill experiments**

AOA-2 MIC against *A. baumannii* ATCC 17978, *P. aeruginosa* PaO1, and *E. coli* ATCC 25922 strains was determined by microdilution assay in 2 independent experiments, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines[3]. MICs of imipenem,

ciprofloxacin, tetracycline, tigecycline, amikacin, ceftazidime, aztreonam, nalidixic acid, chloramphenicol, and colistin alone or in combination with AOA-2 or SXV4 (125 µg/mL) against ATCC 17978, Δ*omp25*, and Δ*omp25*-pwH1266-*omp25*, and MIC of colistin alone or in combination with AOA-2 against Col-S and Col-R standard and clinical isolates were also determined.

Time-kill kinetic assays of ATCC 17978, Δ*omp25*, Δ*omp25*-pwH1266-*omp25* or 11 strains were conducted on Moeller Hinton Broth cation-adjusted in presence of AOA-2 (0, 12.5 or 125 µg/ml), SXV4 (125 µg/ml), colistin (sub-MIC) alone or in combination with AOA-2, SXV4 or colistin were performed in duplicate as previously described[4]. Moreover, in some conditions AOA-2, SXV4 or colistin was added for second time 4 h after bacterial addition. Drugs free broth was evaluated in parallel as a control, and cultures were incubated at 37 °C. Viable counts were determined by serial dilution at 0, 2, 4, 8, and 24 h after adding the AOA-2, SXV4 or colistin, and plating 100 µL of control, test cultures, or dilutions at the indicated times onto sheep blood agar plates. Plates were incubated for 24 h and, after colony counts, the $\log_{10}$ of viable cells (CFU/mL) were determined.

Biofilm

An abiotic solid surface biofilm formation assay was performed as described previously[1]. In brief, we used an overnight culture of 2 standards and 8 clinical isolates of *A. baumannii*, 1 standard and 8 clinical isolates of *P. aeruginosa*, and 1 standard and 6 clinical isolates of *E. coli* diluted 1:1 in fresh Luria Bertani (LB) in 96 well plates that were incubated in presence or not of 0.25 mg/mL AOA-2 without shaking at 37°C during 24 h. Biofilm was stained with crystal violet 0.4% (v/v) and quantified at 580 nm after solubilization with ethanol 95%.

Animals

Immunocompetent C57BL/6 female mice (16-18 g) were obtained from University of Seville facility; they had a sanitary status of murine pathogen free and were assessed for genetic authenticity. Animals were housed in regulation cages with food and water ad libitum. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals[3]. The protocol was approved by the Committee on the Ethics of Animal

Experiments of the University Hospital of Virgen del Rocío, Seville (2012PI/238). All surgery was performed under sodium thiopental anaesthesia, and all efforts were made to minimize suffering.

AOA-2 *in vivo* toxicity

The Reed and Munch method[5] was used. Groups of 6 mice were intraperitoneal (i.p.) inoculated with a single dose of AOA-2 (obtained from human brain, Sigma), starting at 10 mg/kg, in 0.5 mL NaCl 0.9%, and the solution were serially duplicated until 50% mortality was reached. The maximum tolerated dose (LD0), the LD50, and LD100 were defined as the concentrations causing 0, 50, and 100% mortality, respectively.

AOA-2 and colistin pharmacokinetics

Serum AOA-2 levels were determined in healthy mice after a single i.p. administration of 10 mg/kg AOA-2 or 10 mg/kg colistin methanesulfonate. After 5, 15, 30, 60,120, 240, 480, and 1440 min, blood was extracted from the periorbital plexuses of the anesthetized mice; three mice were used for each time point. The AOA-2, colistin A and B, and their prodrugs levels were determined using a HPLC-tandem mass spectrometry (LC-MS/MS). The maximum concentration in serum (Cmax; reported in mg per liter), the area under the concentration-time curve from time zero to ∞ ($AUC_{0-\infty}$; reported inmg-min per liter), and the terminal half-life ($t_{1/2}$; reported in min) were calculated using a computer-assisted method[6].

***A. baumannii* peritoneal sepsis model**

Murine peritoneal sepsis models with *A. baumannii* ATCC 17978, *P. aeruginosa* PaO1, and *E. coli* ATCC 25922 strains were established by i.p. inoculation of the bacteria[7]. Briefly, mice were inoculated with 0.5 mL of the bacterial suspension, which was incubated for 20-24 h in LB at 37°C and mixed in a 1:1 ration with a saline solution containing 10% (w/v) porcine mucin (Sigma, Spain). The minimal bacterial lethal dose 100 (MLD100), LD50 and LD0 were determined by inoculating various groups of mice (6 mice per group) with decreasing amounts of *A. baumannii* ATCC 17978, *P. aeruginosa* PaO1, and *E. coli* ATCC 25922 strains inoculum from 8.5 to 2.3, 9.11 to 3.3, 9.13 to 3.47Log CFU/mL, respectively, and monitoring the survival of the mice for 7 days.

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Therapeutic effect of AOA-2 in murine model of peritoneal sepsis

A murine peritoneal sepsis model with *A. baumannii* ATCC 17978, *P. aeruginosa* PaO1, and *E. coli* ATCC 25922 strains was established by i.p. inoculation of the bacteria. Briefly, animals were infected i.p. with 0.5 mL of the MLD100 of *A. baumannii* ATCC 17978, *P. aeruginosa* PaO1, or *E. coli* ATCC 25922 strains mixed 1:1 with 10% porcine mucin. AOA-2 therapy was established as a treatment 2 h after bacterial inoculation. 56 mice were randomly ascribed to the following groups: 1). controls (without treatment), and 2). AOA-2 administered at 10 mg/kg/d i.p. 2 h after bacterial inoculation with each strain. Mortality was recorded over 72 h. After death or sacrifice of the mice at the end of the experimental period, aseptic thoracotomies were performed, and blood samples were obtained by cardiac puncture for qualitative blood cultures. The spleen and lungs were aseptically removed and homogenized (Stomacher 80; Tekmar Co., USA) in 2 mL of sterile NaCl 0.9% solution. Ten-fold dilutions of the homogenized spleen and lungs were plated onto Sheep blood agar for quantitative cultures (Log10 CFU/g of spleen or lung).

Therapeutic effect of AOA-2 in combination with colistin in murine model of peritoneal sepsis

A murine peritoneal sepsis model was established by i.p. inoculation of *A. baumannii* ATCC 17978 or 11 strains as described above. Sixty-six mice were randomly ascribed to the following groups: 1) controls (without treatment), 2) colistin administered i.p. at 10 mg/kg/24 h for 72 h, and 3) AOA-2 administered at 10 mg/kg/d i.p. 2 h after bacterial inoculation, and colistin administered i.p. at 10 mg/kg/24 h for 72 h. The mortality, bacteremia and bacterial burden were determined as indicated above.

Analysis of OMPs by SDS-PAGE

Bacterial cells were grown in LB to the logarithmic phase, incubated with AOA-2 for 4 h, and were lysed by sonication. Outer broth membrane proteins (OMPs) were extracted with sodium lauroylsarcosinate (Sigma, Spain) and recovered by ultracentrifugation as described previously[2]. The OMP profiles were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% SDS gels and 6 µg protein of OMPs, followed by

Simply Blue SafeStain gel (Invitrogen, Spain).For peptide mass fingerprinting, a Simply Blue SafeStain band representing an unidentified protein with 25 kDa MW was excised from SDS-PAGE gel and sent for MALDI-TOF-TOF (MS-MS/MS) analysis. Data obtained from peptide mass spectroscopy fingerprinting were matched against the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) using the Mascot program.

Generation of Omp25 knockout from *A. baumannii* ATCC 17978 wt

To construct an omp25 knockout from *A. baumannii* ATCC 17978 wt, an internal *omp25*400-bp fragment obtained by PCR amplification with the primers Omp25F and omp25R (Table S1) was cloned into pGEM-T to give plasmid omp25-pGEM-T by using T4 DNA ligase. The resulting construct incorporated into *E. coli* Top10 was purified and electroporated into ATCC 17978 wt to knock out the *omp25* gene. Transformants were selected on LB agar plates containing 80 µg/mL ticarcillin. The *omp25* gene disruption within the resulting strain, designated Δ*omp25*, was confirmed by PCR using a combination of primers matching the upstream region of *omp25* gene and the pGEM-T Easy vector.

Complementation of Δ*omp25* strain

To complement Δ*omp25* strain, the *omp25* gene was amplified with the EcoRI-omp25F and EcoRI-omp25R primers (Table S1) from the ATCC 17978 wt genome and cloned into the EcoRI restriction site of the pwH1266 vector yielding the pwH1266-*omp25* plasmid. pwH1266-*omp25* was transformed into Top10 and electroporated into Δ*omp25*strain. Transformants were selected on LB agar plates containing 30 µg/mL tetracycline and confirmed by PCR with the pBR322-4361F and pBR322-116R primers (Table S1).

Real-time RT-PCR studies

The 11 standard and clinical Col-S strains and the 16 clinical Col-R isolates in presence or absence of AOA-2 (125 µg/ml) were analyzed for the expression of *omp25* gene. DNase-treated bacterial RNA was isolated following the protocol of Trizol Max Bacterial Enhancement Reagent kit (Trizol Max kit; Ambion) from cultures grown to obtain DO = 0.6. Real-time RT-PCR was performed with anCFX96 Real Time PCR Detection System (BioRad, Spain). The concentrations of primers and probes, given in Table S1 were used in this study. Samples were

run in triplicate, and the use of controls without reverse transcriptase confirmed the absence of contaminating DNA in the samples. A total of 35 ng of RNA was used in the target gene studies. The expression of each gene was normalized to that of a ribosomal housekeeping gene. The relative expression of each target gene was then calibrated against the corresponding expression *by A. baumannii* ATCC 17978 (whose expression was set equal to 1.0), which served as the control.

Table S1. Bacterial Strains, Plasmids, and Primers Used in This Study.

Strains, plamids and primers	Relevant features and use	References
A. baumannii ATCC 17978 wt	Reference strain isolated in a fatal meningitis infant	8
A. baumannii ATCC 19606	Reference strain isolated from urine, United States, 1948.	9
A. baumannii C4, C5, C12	Colonizing MDR strains isolated from tracheobronchial aspirate of mechanically ventilated individual adult patients	10
A. baumannii IB1, IB2	Bacteremic MDR strains isolated from tracheobronchial aspirate of mechanically ventilated individual adult patients	10
A. baumannii HC1, HC2	Bacteremic MDR strains isolated from blood of mechanically ventilated individual adult patients	10
A. baumannii 77	MDR strain isolated from respiratory sample.	11
A. baumannii 42	Colonizing MDR strain isolated from wound exaudate	12
A. baumannii 1, 10, 11, 14, 16, 17, 19. 20, 21R, 21P, 24, 99, 113-16	Panresistant strains isolated from different biological samples	13
A. baumannii 345	Colistin R	12
A. baumannii RC64	an *in-vitro*-derived colistin-resistant mutant from ATCC 19606	14
A. baumannii CS01	isolated from the cerebrospinal fluid of a patient with meningitis prior to colistin treatment	15
A. baumannii CR17	Colistin-resistant derivative of CS01 which was isolated from cerebrospinal fluid 9 days after initiation of treatment with colistin	15
P. aeruginosa PaO1	Reference strain	16
P. aeruginosa 15, 160	Bacteremic MDR strains isolated from blood	17
P. aeruginosa 17, 61, 127, 142, 184, 204	Bacteremic strains isolated from blood	17
E. coli ATCC 25922	Reference strain. Serotype O6, Biotype 1	18
E. coli 12-69, 7-9, 7-2, 11-51-2, 5-38, 12-74	Bacteremic strains isolated from blood in the Virgen Macarena University hospital of Seville.	--
A. baumannii omp25:pGEM-T	Derived from *A. baumannii* ATCC 17978 wt. Omp25 mutant obtained.	This study
E. coli Top10	Used for recombinant DNA methods	Invitrogen
pGEM-T	Suicide plasmid for *A. baumannii*. Amp^R	Promega
pWH1266	*Escherichia coli*-Acinetobacter shuttle plasmid. $Tet^R Amp^R$	19
omp25intF	5-CAAACACGTAATGCACCGCT-3	This study
omp25intR	5-CGTCAAGGTCACCAAACGCA-3	This study
omp25extF	5-GCACATGGTTACAGTCCAGT-3	This study
omp25extR	5-GGTAATGTGAGCTTACAGCT-3	This study
M13F	5-GTAAAACGACGGCCAGT-3	1
M13R	5-CAGGAAACAGCTATGAC-3	1
EcoRI-omp25F	5-GACTAGGAATTCGCACATGGTTACAGTCCAGT-3	This study
EcoRI-omp25R	5-GACTAGGAATTCGGTAATGTGAGCTTACAGCT-3	This study
pBR322-4361F	5-AGTGCCACCTGACGTCTAAGAAAC-3	This study
pBR322-116R	5-GGATGACGATGAGCGCATTGTTAG-3	This study

Table S2. Docking score of all the peptides from the EXORIS library.

Cyclic peptide	Docking score
&Arg-D-Pro-Trp-Arg-D-Pro-Trp&	-10.8
&Trp-D-Pro-Arg-Trp-D-Pro-Arg&	-10.6
&Arg-Pro-Trp-Arg-Pro-Trp&	-10.3
&Trp-Pro-Arg-Trp-Pro-Arg&	-10.0
&Trp-Pro-D-Arg-Trp-Pro-D-Arg&	-9.2
&Arg-Pro-D-Trp-Arg-Pro-D-Trp&	-8.9
&Arg-D-Pro-D-Ile-Arg-D-Pro-Ile&	-8.5
& Trp- D-Pro-Arg-Trp-D-Pro-Arg&	-8.5
&Arg-D-Pro-Ile-Arg-D-Pro-Ile&	-8.2
&Ser-D-Pro-Trp-Ser-D-Pro-Trp&	-7.5
&Ser-D-Pro-D-Trp-Ser-D-Pro-Trp&	-7.4
&Ser-D-Pro-D-Ile-Ser-D-Pro-Ile&	-7.3
&Ser-D-Pro-Ile-Ser-D-Pro-Ile&	-7.2
&D-Ser-Pro-Ala-D-Ser-Pro-Ala&	-7.1
&Glu-D-Pro-Trp-Glu-D-Pro-Trp&	-7.0
&Ile-D-Pro-D-Trp-Ile-D-Pro-Trp&	-6.8
&Ser-D-Pro-Ala-Ser-D-Pro-Ala&	-6.8
&Ile-D-Pro-Trp-Ile-D-Pro-Trp&	-6.7
&D-Arg-Pro-Trp-D-Arg-Pro-Trp&	-6.1
&Ser-D-Pro-Glu-Ser-D-Pro-Glu&	-6.1
&Glu-D-Pro-D-Trp-Glu-D-Pro-Trp&	-6.1
&Ser-D-Pro-D-Glu-Ser-D-Pro-Glu&	-6.1
&Ser-Pro-Ala-Ser-Pro-Ala&	-5.6
&Glu-D-Pro-Ile-Glu-D-Pro-Ile&	-4.6
&Glu-D-Pro-D-Ile-Glu-D-Pro-Ile&	-4.4
&Gly-Pro-Ala-Gly-Pro-Ala&	-2.9

& symbol means cyclic peptides

Table S3. List of hexapeptides and peptide control of EXORIS computationally screened library.

N°	EXORIS code	Name	Cyclic peptide	Purity (%)	Rational
1	600-032C	AOA-1	&Arg-D-Pro-Trp-Arg-D-Pro-Trp&	96	Candidate (Trp, Arg)
2	600-012C	AOA-2	&Trp-D-Pro-Arg-Trp-D-Pro-Arg&	92	Sequence change
3	600-032D	AOA-3	&Arg-Pro-D-Trp-Arg-Pro-D-Trp&	100	Different stereoisomer
4	600-032B	AOA-4	&D-Arg-Pro-Trp-D-Arg-Pro-Trp&	95	Different stereosiomer
5	600-034	AOA-5	&Ser-D-Pro-Trp-Ser-D-Pro-Trp&	100	Arg replaced by Ser
6	600-033	AOA-6	&Glu-D-Pro-Trp-Glu-D-Pro-Trp&	95	Arg replaced by Glu
7	--	SXV4	Ac-Trp-D-Pro-Arg-Trp-D-Pro-Arg-OH	100	Negative Control

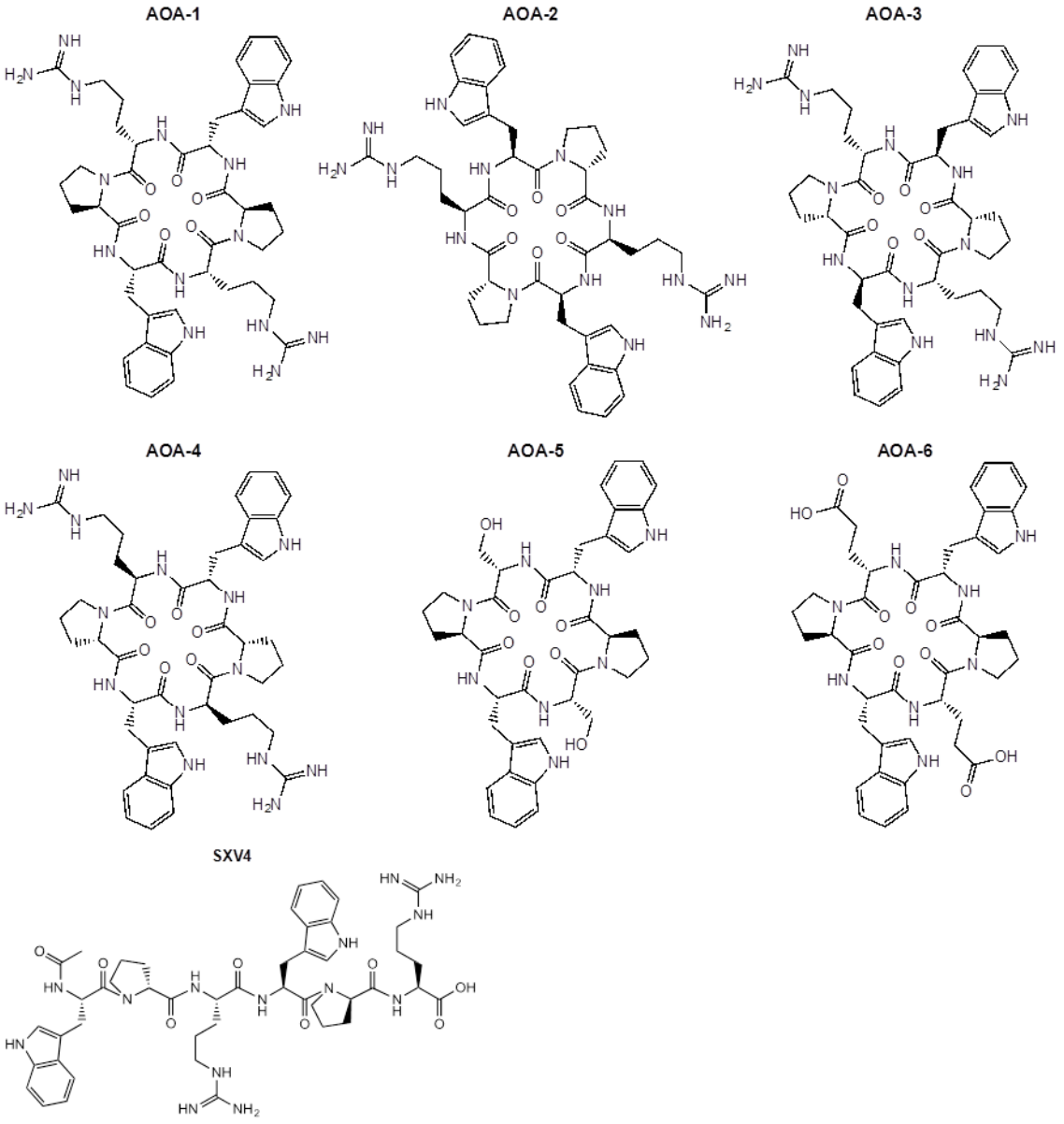

Fig. S1. Structure of six hexapeptides and peptide control (SXV4) used in the study.

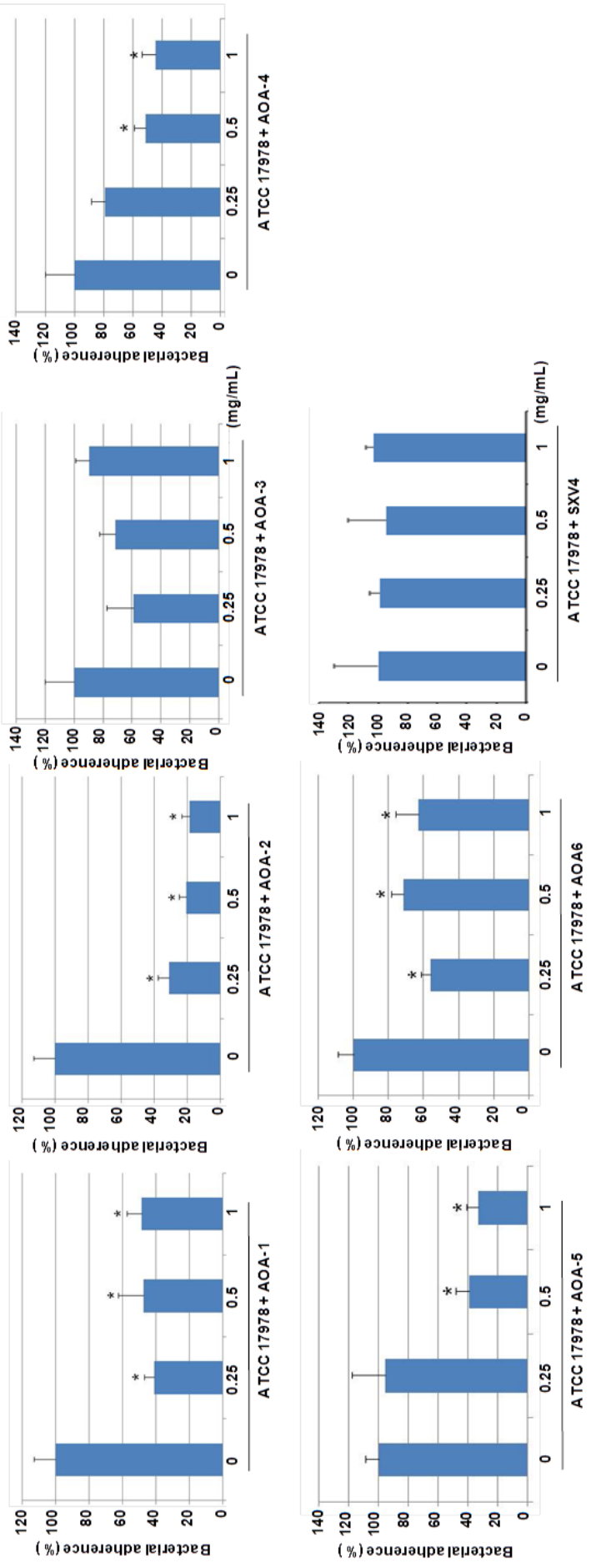

Fig. S2. OmpA inhibitor hexapeptides reduce *A. baumannii* adherence to A549 cells. A549 cells were infected with 10^8 CFU/mL of ATCC 17978 strain during 2 h in presence of AOA-1, AOA-2, AOA-3, AOA-4, AOA-5, AOA-6, or SXV4 (0, 0.25, 0.5, or 1 mg/mL). Representative results of three independent experiments are shown; data are means ± SEM. * $P<0.05$: between untreated and treated groups.

Table S4. Percentages of A549 cellular toxicity assessed through mitochondrial reduction activity (MTT assay) in presence of anti-OmpA hexapeptides and control peptide (SXV4).

	Concentration (mg/mL)		
Peptides	**0.25**	**0.5**	**1**
AOA-1	98.89 ± 0.41	99.69 ± 0.26	99.7 ± 0.38
AOA-2	98.09 ± 0.27	98.33 ± 0.4	98.48 ± 0.55
AOA-3	99.27 ± 0.21	99.7 ± 0.14	99.36 ± 0.3
AOA-4	99.47 ± 0.39	99.77 ± 0.17	100.13 ± 0.18
AOA-5	98.86 ± 0.08	100.05 ± 0.39	100.14 ± 0.46
AOA-6	98.31 ± 0.35	99.14 ± 0.44	99.38 ± 0.34
SXV4	98.17 ± 0.6	98.79 ± 0.28	98.55 ± 0.21

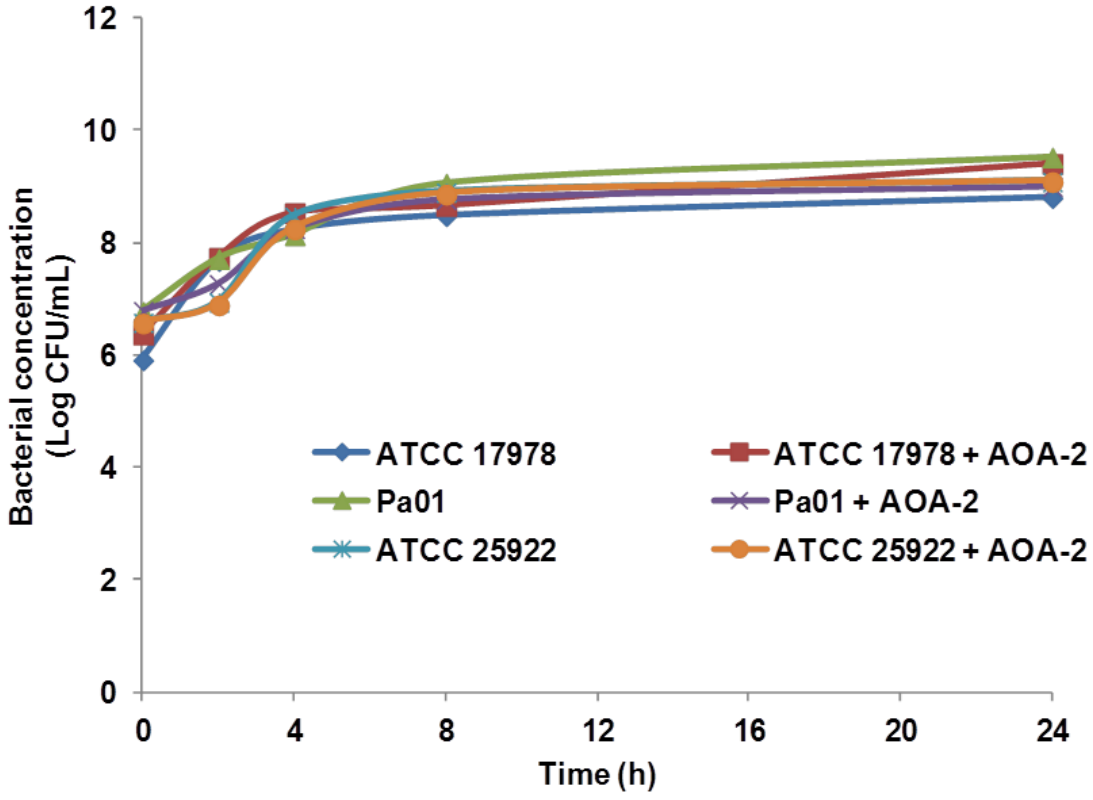

Fig. S4. Time kill curves of *A. baumannii* ATCC 17978, *P. aeruginosa* PaO1, and *E. coli* ATCC 25922 strains in presence of 125 µg/mL AOA-2.

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Table S5. Effect of AOA-2 (125 µg/mL) on biofilm formation by standard strains and clinical isolates of *A. baumannii*, *P. aeruginosa* and *E. coli*.

Pathogen	Strain	without AOA-2	with AOA-2
A. baumannii	ATCC 17978	1.16 ± 0.14	0.54 ± 0.23
	ATCC 19606	0.98 ± 0.1	0.61 ±0.1
	77	0.98 ± 0.05	0.73 ± 0.08
	C4	1.04 ± 0.003	0.5 ± 0.006
	C5	0.9 ± 0.05	0.42 ± 0.06
	C12	0.99 ± 0.003	0.51 ± 0.02
	IB1	1.12 ± 0.11	0.58 ± 0.04
	IB2	1.08 ± 0.02	0.43 ± 0.06
	HC1	0.83 ± 0.06	0.41 ± 0.1
	HC2	0.97 ± 0.11	0.52 ± 0.1
P. aeruginosa	PaO1	0.77 ± 0.02	0.53 ± 0.05
	15	0.86 ± 0.02	0.2 ± 0.01
	17	0.84 ± 0.02	0.18 ± 0.002
	61	0.57 ± 0.01	0.21 ± 0.01
	127	0.71 ± 0.02	0.18 ± 0.01
	142	0.93 ± 0.03	0.27 ± 0.01
	160	0.91 ± 0.02	0.17 ± 0.01
	184	1.00 ± 0.12	0.41 ± 0.07
	204	0.71 ± 0.02	0.37 ± 0.01
E. coli	ATCC 10536	0.68 ± 0.019	0.2 ± 0.02
	12-69	0.41± 0.02	0.19 ± 0.02
	7-9	0.27 ± 0.03	0.09 ± 0.01
	7-2	0.26 ± 0.03	0.13 ± 0.01
	11-51-2	0.32 ± 0.01	0.21 ± 0.007
	5-38	0.17 ± 0.01	0.09 ± 0.006
	12-74	0.23 ± 0.01	0.18 ± 0.007

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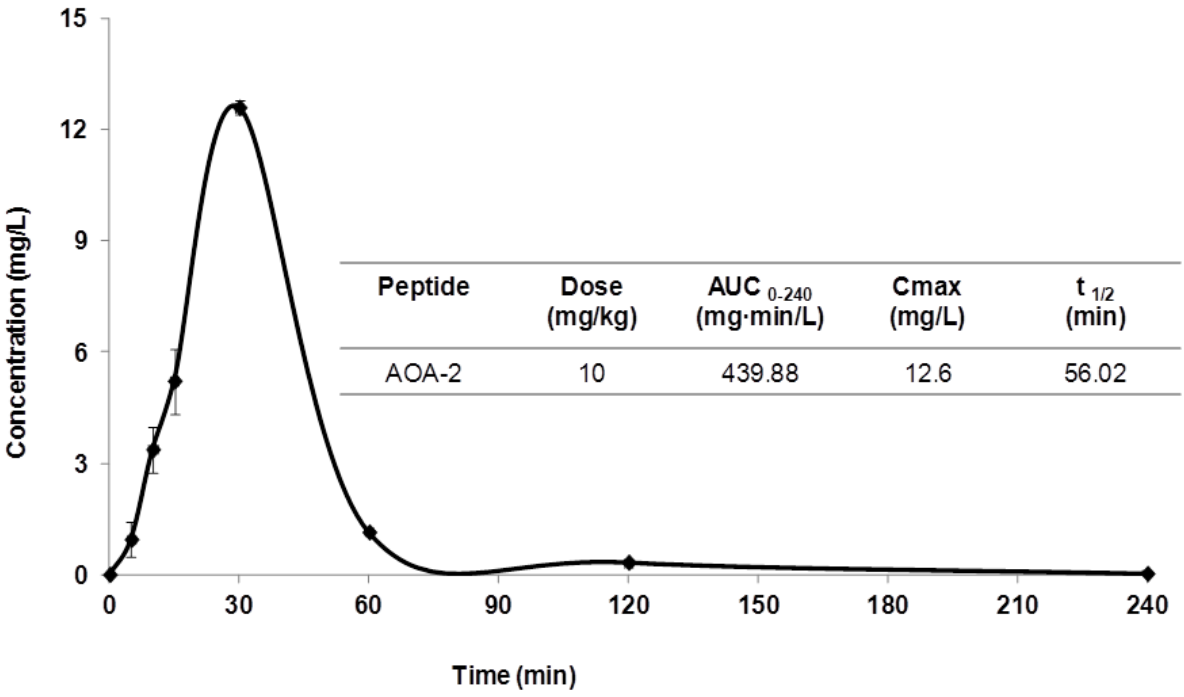

Peptide	Dose (mg/kg)	AUC $_{0-240}$ (mg·min/L)	Cmax (mg/L)	$t_{1/2}$ (min)
AOA-2	10	439.88	12.6	56.02

Figure S5. Serum AOA-2 pharmacokinetic parameters. AOA-2 was intraperitoneally administered to mice at a dose of 10 mg/kg, and serum AOA-2 concentrations were analyzed for 240 min. C_{max}, maximum concentration in serum; $t_{1/2}$, elimination half-time; AUC_{0-240}, area under the concentration-time curve from time 0 to 240 min.

Table S6. *In vivo* toxicity of AOA-2. Each group of six mice was administered 0.5 ml of AOA-2 at 10, 20, 40, 80 or 160 mg/kg. Mice survival was monitored for a one week and lethal doses (LD) 0, 50 and 100 were calculated.

	LD0	LD50	LD100
MV5 (mg/kg)	40	85	160

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Table S7. MICs of different antimicrobial agents for *A. baumannii* ATCC 17978 in presence or absence of AOA-2.

Antimicrobial agents	MIC (µg/ml)		Difference in susceptibility (fold)[b]
	ATCC 17978	ATCC 17978 + AOA-2[a]	
Imipenem	0.125	0.125	1
Ciprofloxacin	0.5	0.5	1
Tetracycline	8	8	1
Tigecycline	0.5	0.5	1
Amikacin	4	4	1
Ceftazidime	8	8	1
Aztreonam	32	8	**4**
Nalidixic acid	32	8	**4**
Chloramphenicol	>256	64	**>4**

a: AOA-2 = 125 µg/mL.
b: Values in bold indicate a difference in susceptibility of greater than two-fold.

Table S8. Determination of colistin MIC in standard strains and clinical isolates of *A. baumannii* in presence or absence of AOA-2 or SXV4.

	Bacterial strains	MIC (µg/ml)			Difference in susceptibility (fold)[b]
		Colistin	Colistin+ AOA-2[a]	Colistin+ SXV4[a]	
Col-S	ATCC 17978	0.5	≤0.015	0.25	**≥33**
	JPAB01	0.0625	0.0625	ND	1
	JPAB01+pJPAB01	0.25	≤0.015	ND	**≥17**
	77	0.5	0.031	ND	**17**
	42	2	≤0.015	ND	**≥133**
Col-R	1	>256	32	ND	**> 8**
	10	>256	64	ND	**> 4**
	11	16	2	16	**8**
	14	8	0.5	ND	**16**
	16	>256	32	ND	**>8**
	17	8	0.5	ND	**16**
	19	>256	>256	ND	>1
	20	>256	128	ND	**> 2**
	21R	>256	128	ND	**> 2**
	22P	>512	>512	**ND**	>1
	24	16	2	ND	**8**
	345	4	≤0.015	ND	**≥267**
	99	512	64	ND	**8**
	113-16	64	4	ND	**16**
Col-S & Col-R	ATCC 19606	0.125	≤0.015	ND	**≥8**
	RC64	64	16	ND	**4**
	CS01	0.0625	≤0.015	ND	**≥4**
	CR17	256	256	ND	1
	CMI 50	32	2	ND	**16**
	CMI 90	512	256	ND	**2**

a: AOA-2 or SXV4 = 125 µg/mL.
b: Values in bold indicate a difference in susceptibility of greater than two-fold.

Table S9. Pharmacokinetic and pharmacodynamics parameters of sub-optimal dose of colistin.

Drug	Dose (mg/kg)	C_{max} (µg/mL)	$T_{1/2}$ (h)	$AUC_{0\text{-}8h}$ (µg.h/mL)	$AUC_{0\text{-}24h}$ (µg.h/mL)	$AUC_{0\text{-}24h}$/MIC
CMS	10	7.9	0.82	8.08	24.23	48.46[a]/1.51[b]
Total colistin	10	1.35	1.1	2.03	6.11	12.22[a]/0.38[b]
Free colistin	10	0.46	1.01	0.64	1.92	3.84[a]/0.12[b]

[a]Colistin MIC of ATCC 17978 strain: 0.5 µg/mL
[b]Colistin MIC of #11 strain: 16 µg/mL

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Chapter I. Article I: additional data

ADDITIONAL DATA

Here we present additional data which are not included in the previous results of the article I, but were important to understand how AOA-2 interacts with OmpA.

First, in order to determine the homology of *A. baumannii*, *P. aeruginosa* and *E. coli* OmpA homologues, we aligned the sequences of these proteins by clustal alignment using the protein database UniProtKB, which is showed in **figure A1**. Then, in collaboration with Dr. Ernest Giralt team in the Institute for Research in Biomedicine (IRB) molecular docking to predict the preferred orientation of the ligand to the protein to form a stable complex was performed. To do so, Glide XP scoring function and molecular docking software from SCHRODINGER package were used. As showed in **figure A2**, we identified *in silico* the 24 amino acids of *A. baumannii* OmpA are in contact with the active ligand AOA-2.

Then, to demonstrate that the inhibitor peptide AOA-2 interacts with OmpA, we reconstituted *E. coli* OmpA into planar lipid bilayers to form ion channels and analyze their activity after the addition of the peptide (**Figure A3**). These experiments were performed in Dr. Thierry Jouenne's Polymères-Biopolymères-Surfaces (PBS) laboratory from the University of Rouen, in France, under the guidance of Dr. Emmannuelle Dé. Briefly, the reconstitution of *E. coli* OmpA was performed in virtually solvent-free planar lipid bilayers, by the method of Montal and Mueller (306), as modified by Saint *et al*. (307). From a diphytanoylphosphocholine (DPhPC, Avanti, Birmingham, USA) solution in hexane, lipid bilayers were formed by the apposition of two monolayers on a 100-μm-diameter hole in a thin Teflon film (10 μm) sandwiched between two half glass cells pretreated with hexadecane/hexane solution (1:40, v/v). Bilayer formation was monitored by the capacitance response and the voltage and current sign conventions are the usual ones. The electrolyte solution was 1 M KCl, 10 mM HEPES, (pH 7.4). The proteins, in 1 % octyl glucopyranoside (OG), were added to the measurement compartments in a symmetric manner. Conductance of OmpA was tested after the addition in the electrolyte solution of increasing concentrations of OmpA inhibitor peptide (AOA-2) and the control peptide (SXV1).

After the addition of OmpA into the electrolytic compartment, we detected current fluctuations when a voltage is applied. Once we had many open channels and tested that

they are stable, we added increasing concentrations of AOA-2 at different time points, meanwhile we recorded the conductance to determine the activity of the channels (**Figure A3**). We verified that the addition of AOA-2 inhibits the conductance of OmpA channels in a concentration-dependent manner. Only 34 % of the channels remained open in the last point, when we reached the maximum concentration chosen of the ligand in the media, which means that OmpA channels were closed or blocked due to the addition of the peptide to the electrolytic media (**Figure A4**). We performed the same experiment with the control peptide SXV1 and its addition showed no difference in the conductance of the channels, even at the highest concentration, with almost a 99 % of open channels.

```
OMP38_ACIBA       1   MKLSRIALATMLVAAPLA---AANAGVTVTPLLLGYTFQDSQHNNGGKDGNLTNGPELQD   57
OMPA_ECOLI        1   MKKTAIAIAVALAGFATVAQAAPKDNTWYTGAKLGWS----QYHDTG--FINNNGPTHEN   54
Q9XAX8_PSEAC      1   ------------------------------------------------------------    0

OMP38_ACIBA      58   DLFVGAALGIELTPWLGFEAEYNQVKGDV-DGASAGAEYKQKQINGNFYVTSDLITKNYD  116
OMPA_ECOLI       55   QLGAGAFGGYQVNPYVGFEMGYDWLGRMPYKGSVENGAYKAQGVQL-----TAKLGYPIT  109
Q9XAX8_PSEAC      1   ------------------------------------------------MKSVIA    6
                                                                       :

OMP38_ACIBA     117   SKIKPYVLLGAGHYKYDFDGVNRGTR-GTSEEGTLGNAGVG-----------AFWR----  160
OMPA_ECOLI      110   DDLDIYTRLGGMVWRADTKSNVYGKNHDTGVSPVFAGGVEYAITPEIATRLEYQWT--NN  167
Q9XAX8_PSEAC      7   ASLVIFTLTGCASIQN-EDGTTKNTA-LYGAGGALAGAVAG-----------ALIGKENR   53
                       .:  :.  *    :   ..   ..    .   .:...

OMP38_ACIBA     161   LNDALSLRTEARATYNADEEFWNYTALAGLNVVLGGHLKPAAPVVEVAPVEPTPVAPQPQ  220
OMPA_ECOLI      168   IGDAHTIGTRP-----------DNGMLSLGVSYRFGQGEAAPVVA------PAPAPAPE  209
Q9XAX8_PSEAC     54   AQGALI-GAAVAGSLGAGYGYYADKQEAELRE----QMKGS-----------GVQVERQ   96
                        .*    :               .    *          :                :

OMP38_ACIBA     221   ELTEDLNMELRVFFDTNKSNIKDQYKPEIAKVAEKLSEYP--NATARIEGHTDNTGPRKL  278
OMPA_ECOLI      210   VQTKHFTLKSDVLFNFNKATLKPEGQAALDQLYSQLSNLDPKDGSVVVLGYTDRIGSDAY  269
Q9XAX8_PSEAC     97   GDEIVIVMPGAITFATGKAEIQPNFANTLNQLAGSFRNYP--DSRLIVTGHTDSVGSYEA  154
                           : :   : *  .*: :: :    : ::  .: :    :.   : *:**  *

OMP38_ACIBA     279   NERLSLARANSVKSALVNEYNVDASRLSTQGFAWDQPIADNKTKE---------GRAMNR  329
OMPA_ECOLI      270   NQGLSERRAQSVVDYL-ISKGIPADKISARGMGESNPVTGNTCDNVKQRAALIDCLAPDR  328
Q9XAX8_PSEAC    155   NELLSQRRAQSVAQF-LRGNGVQTDRIEVIGAGPNQPVASNATAE---------GRAQNR  204
                      *: **  **:** .      .: :.::.. * . .:*::.*   :           * :*

OMP38_ACIBA     330   RVFATITGSRTVVVQPGQEAAAPAAAQ                                 356
OMPA_ECOLI      329   RVEIEVKGIKDVVTQPQA---------                                 346
Q9XAX8_PSEAC    205   RVEIKLAPRAVQQA--SR---------                                 220
                      **   :       .
```

Figure A1. Clustal alignment of OmpA homologs from *A. baumannii*, *P. aeruginosa* and *E. coli*. The OmpA protein from *A. baumannii* (OMP38_ACIBA, accession number Q6RYW5 at UniProtKB) was aligned with *E. coli* OmpA (OMPA_ECOLI, accession number P0A910) and *P. aeruginosa* OmpA (Q9XAX8_PSEAC, accession number Q9XAX8).

L31	Y33	Q36	D37	Q39	H40	Y130	Y132	E146	E147	G148	L150	R172

A173	T174	N176	A177	D178	E179	E180	W182	N183	Y184	T185

Figure A2. *In silico* identification of amine acids of *A. baumannii* OmpA that contact with the active ligand AOA-2.

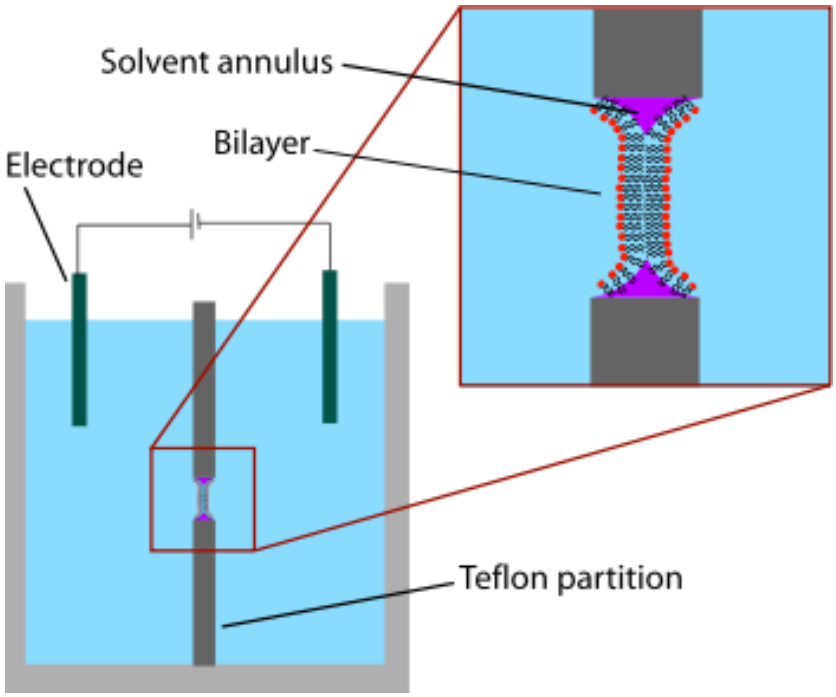

Figure A3. Planar lipid bilayer scheme. Representative illustration of planar lipid bilayer formed in a Teflon film.

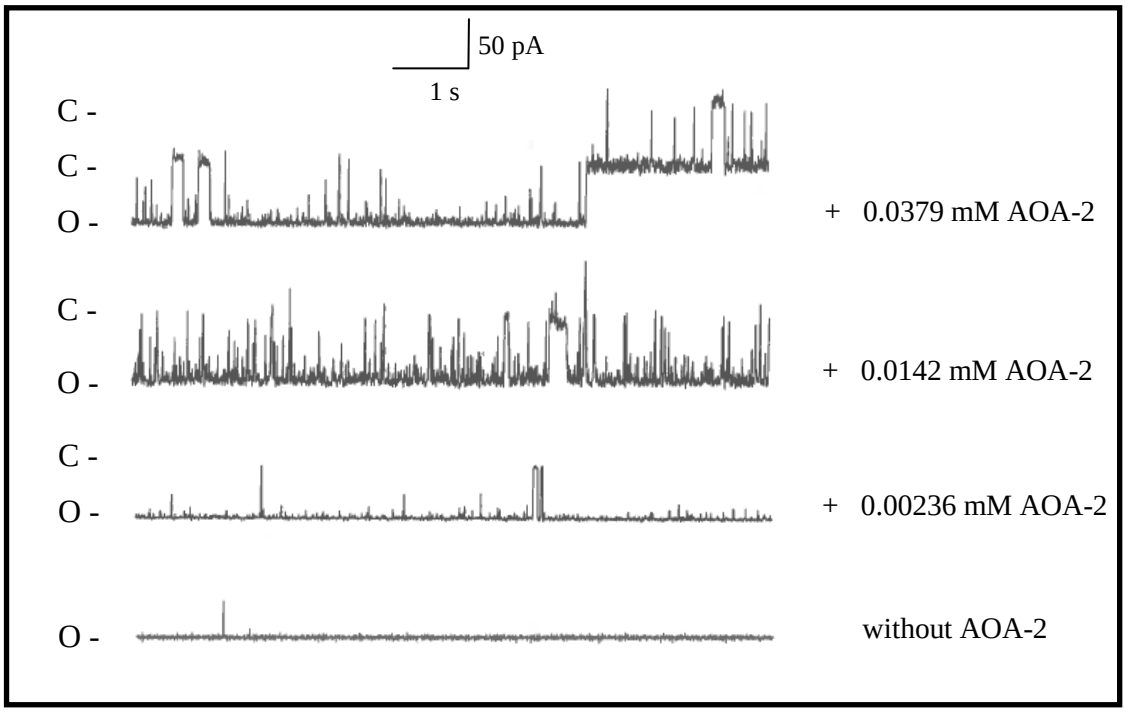

Figure A4. OmpA channel closure by AOA-2 peptide. Recordings of OmpA channel activity after reconstitution in a DPhPC bilayer bathing by 1M KCl, pH 7.4, applied voltage: -60 mV. Addition of increasing concentrations of the peptide induced an increasing number of closing events and progressive channel-blocking. First addition: 10 µL of AOA-2 1/10. Second addition: 10 µL of AOA-2 ½. Third addition: 10 µL of AOA-2 concentrated. O: open channels; C: closed channels.

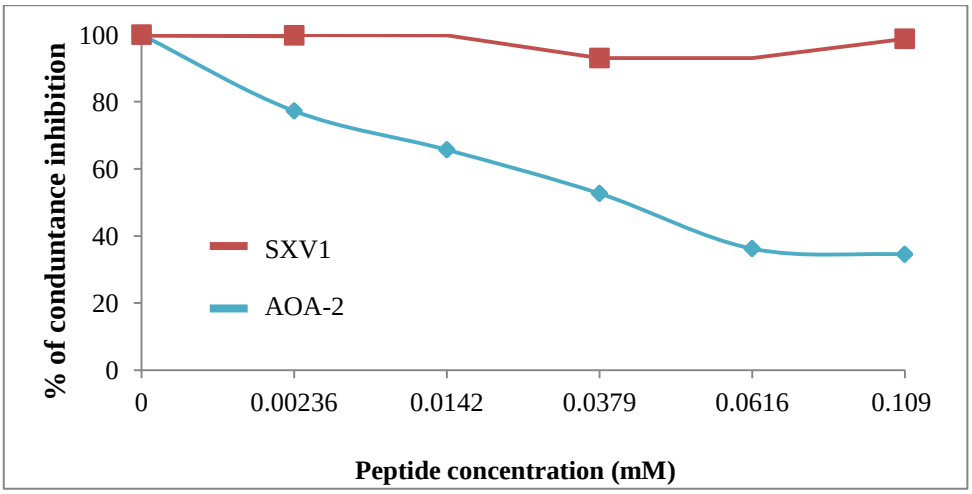

Figure A5. OmpA conductance by increasing concentrations of AOA-2 or SXV1. Planar lipid bilayer reconstitution was performed for *E. coli* OmpA. Three experiments were performed for each concentration tested. Increasing concentrations of AOA-2 (blue) and control peptide SXV1 (red) were added to the electrolyte solution to test the inhibition of OmpA channels opening.

2. Chapter II

Chapter II. Article II

2.1. Article II. Efficacy of lysophosphatidylcholine in combination with antimicrobial agents against *Acinetobacter baumannii* in experimental murine peritoneal sepsis and pneumonia models

Due to the significant increase in antimicrobial resistance of *A. baumannii*, immune system stimulation to block the infection progression could be a therapeutic alternative adjuvant to antimicrobial treatment. LPC is an immunomodulator that is involved in the immune cells recruitment and activation. Previously, we have demonstrated in experimental murine models of peritoneal sepsis and pneumonia caused by *A. baumannii*, that LPC preemptive treatment improves bacterial clearance in spleen and lung, reduces bacteremia and increases mice survival to 40 %. Consequently, we suggested the application of LPC as an adjuvant pretreatment in combination with antimicrobial agents to treat *A. baumannii* infections, including MDR strains. Therefore, we aimed to evaluate the efficacy of LPC in combination with colistin, tigecycline or imipenem in murine peritoneal sepsis and pneumonia models.

A. baumannii susceptible strain to colistin, tigecycline and imipenem, and MDR strain susceptible to colistin and resistant to tigecycline and imipenem were used. Pharmacokinetics and pharmacodynamics parameters of colistin, tigecycline and imipenem, and minimal lethal dose (MLD) of both strains were determined. In murine experimental models of peritoneal sepsis and pneumonia, mice were pretreated with LPC at 25 mg/kg, infected with MLD of susceptible or MDR strain, and combined or not with colistin (20 mg/kg/8 h), tigecycline (5 mg/kg/12 h) during 72 h or imipenem (30 mg/kg/4 h) during 24 h. Bacterial load in spleen and lungs, bacteremia and mice survival were analyzed. Furthermore, the levels of pro- and anti-inflammatory cytokines, TNF-α and IL-10, respectively, were determined by enzyme-linked immunosorbent assay (ELISA) after induction of peritoneal sepsis model by both *A. baumannii* strains.

The results of this study showed that LPC pretreatment in combination with colistin, tigecycline or imipenem improves the evolution of both peritoneal sepsis and pneumonia caused by *A. baumannii* susceptible and MDR strains. Moreover, the host inflammatory response induced by peritoneal sepsis caused by *A. baumannii* susceptible strain is different from that caused by the MDR strain.

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Efficacy of Lysophosphatidylcholine in Combination with Antimicrobial Agents against *Acinetobacter baumannii* in Experimental Murine Peritoneal Sepsis and Pneumonia Models

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Immune response stimulation to prevent infection progression may be an adjuvant to antimicrobial treatment. Lysophosphatidylcholine (LPC) is an immunomodulator involved in immune cell recruitment and activation. In this study, we aimed to evaluate the efficacy of LPC in combination with colistin, tigecycline, or imipenem in experimental murine models of peritoneal sepsis and pneumonia. We used *Acinetobacter baumannii* strain Ab9, which is susceptible to colistin, tigecycline, and imipenem, and multidrug-resistant strain Ab186, which is susceptible to colistin and resistant to tigecycline and imipenem. Pharmacokinetic and pharmacodynamic parameters for colistin, tigecycline, and imipenem and the 100% minimal lethal dose (MLD_{100}) were determined for both strains. The therapeutic efficacies of LPC, colistin (60 mg/kg of body weight/day), tigecycline (10 mg/kg/day), and imipenem (180 mg/kg/day), alone or in combination, were assessed against Ab9 and Ab186 at the MLD_{100} in murine peritoneal sepsis and pneumonia models. The levels of pro- and anti-inflammatory cytokines, i.e., tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10), were determined by enzyme-linked immunosorbent assay (ELISA) for the same experimental models after inoculating mice with the MLD of both strains. LPC in combination with colistin, tigecycline, or imipenem markedly enhanced the bacterial clearance of Ab9 and Ab186 from the spleen and lungs and reduced bacteremia and mouse mortality rates ($P < 0.05$) compared with those for colistin, tigecycline, and imipenem monotherapies. Moreover, at 4 h post-bacterial infection, Ab9 induced higher TNF-α and lower IL-10 levels than those with Ab186 (4 μg/ml versus 3 μg/ml [$P < 0.05$] and 2 μg/ml versus 3.4 μg/ml [$P < 0.05$], respectively). LPC treatment combined with colistin, tigecycline, or imipenem modestly reduced the severity of infection by *A. baumannii* strains with different resistance phenotypes compared to LPC monotherapy in both experimental models.

Acinetobacter baumannii is a Gram-negative coccobacillus with high clinical relevance due to the different severe nosocomial infections that it causes, mainly in intensive care units, and its capacity to develop resistance to most of the antimicrobial agents used in clinical practice (1).

A multidrug resistance pattern is commonly observed for *A. baumannii* isolates, raising the threat of impossible-to-treat infections (2). These multidrug-resistant (MDR) isolates are generally susceptible to polymyxins (colistin and polymyxin B) and resistant to imipenem and tigecycline (3, 4). The limited antimicrobial alternatives for the treatment of severe infections by MDR *A. baumannii* make the search for other therapeutic options urgent. Polymyxins have been used as a last resort to treat infections by MDR *A. baumannii*. In humans, suboptimal and optimal doses of colistin to treat ventilator-associated pneumonia due to MDR *A. baumannii* prevent mortality in only 38.1% and 62.5% of cases, respectively (5, 6). Recently, in a clinical trial at our hospital, treatment with the optimized dose of colistin, 3 million units (MU)/8 h, prevented mortality in only 50% of patients (4 of 8 patients) with *A. baumannii* infection (unpublished data). Moreover, the use of colistin to treat infections with *A. baumannii* clinical isolates susceptible to it protected only 33 to 40% of mice from mortality (7, 8).

Lysophosphatidylcholine (LPC) is a major component of phospholipids involved in the recruitment and stimulation of immune cells and the elimination of dead eukaryotic and prokaryotic cells during infection (9–13). We previously successfully demonstrated the efficacy of LPC as a preemptive treatment in murine peritoneal sepsis and pneumonia experimental models of infection caused by susceptible *A. baumannii* strains (14). From this study, we suggested the clinical application of LPC as an adjuvant pretreatment in combination with antimicrobial agents for the treatment of infections by *A. baumannii*, including MDR strains (14).

Currently, there are no data regarding the efficacy of LPC in combination with antimicrobials, such as colistin, tigecycline, and imipenem, all of which are commonly used in severe *A. baumannii* infections. Thus, the aim of this study was to evaluate the efficacy

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of LPC in combination with these antibiotics in a murine experimental model of peritoneal sepsis caused by two clinical isolates of *A. baumannii*: one susceptible to colistin, tigecycline, and imipenem and the other susceptible only to colistin and resistant to tigecycline and imipenem.

MATERIALS AND METHODS

Bacterial strains. An *A. baumannii* clinical strain (Ab9) susceptible to colistin, tigecycline, and imipenem, isolated from a wound surgical exudate, and an MDR *A. baumannii* clinical strain (Ab186) susceptible to colistin and resistant to tigecycline and imipenem, isolated from blood cultures, were used for this study, in addition to a reference strain (ATCC 17978) isolated from an infant with fatal meningitis (15). The Ab9 and Ab186 strains were from the REIPI-GEIH 2010 collection (4) and were of types ST297 and ST2 (international clone II), respectively.

Antimicrobial agents and reagents. For the *in vitro* assays, standard laboratory powders of the following antimicrobials were used: colistin (Sigma, Spain), tigecycline (Sequoia Research Products Ltd., United Kingdom), and imipenem (Sigma, Spain). For the *in vivo* experiments, clinical formulations of the following antimicrobials were used: colistin methanesulfonate (CMS) (G.E.S., Spain), tigecycline (Pfizer, Spain), and imipenem (Merck Sharp & Dohme, Spain). The anesthetic was 5% (wt/vol) sodium thiopental administered intraperitoneally (i.p.) (B. Braun Medical S.A., Spain).

***In vitro* susceptibility testing.** MICs were determined by broth microdilution assay according to standard CLSI recommendations (16), as previously described (17). *Escherichia coli* ATCC 25922 was used as a control strain.

Animals. Immunocompetent C57BL/6 female mice weighing 18 to 20 g (Production and Experimentation Animal Center, University of Seville, Seville, Spain) were used. Animals were housed in regulation boxes and given free access to food and water. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (18). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University Hospital of Virgen del Rocío of Seville, Spain (approval 2014PI/014).

Antimicrobial pharmacokinetic (PK) and pharmacodynamic (PD) parameters. Serum antimicrobial concentrations were determined for groups of 30 healthy mice following single doses of i.p. colistin methanesulfonate (20 mg/kg of body weight), subcutaneous (s.c.) tigecycline (5 mg/kg), or intramuscular (i.m.) imipenem (30 mg/kg).

For sets of three animals 0, 5, 10, 15, 30, 60, 90, 120, 240, 480, and 1,440 min after drug administration, blood samples were obtained from anesthetized mice via the periorbital plexus. Concentrations of colistins A and B and their prodrugs, colistin methanesulfonate A and colistin methanesulfonate B, as well as tigecycline and imipenem, were measured using high-pressure liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) (19–21). The maximum concentration of drug in serum (C_{max}), the area under the concentration-time curve from 0 h to 24 h (AUC_{0-24}), the $AUC_{0-\infty}$ for the free, unbound fraction of drug ($fAUC_{0-24}$), the half-life ($t_{1/2}$), the cumulative percentage of a 24-h period that the drug concentration exceeds the MIC under steady-state pharmacokinetic conditions ($\%T_{MIC}$), the AUC over 24 h in the steady state divided by the MIC for the free, unbound fraction of drug (*f*AUC/MIC), and the AUC/MIC value were obtained by a computer-assisted method (22). Final dosing for the *in vivo* efficacy experiments was adjusted to achieve values for the pharmacodynamic parameters *f*AUC/MIC (for free colistin) and AUC/MIC (for total tigecycline) similar to the ranges (17.5 to 22.5 and 18.5 to 37) for colistin and tigecycline, respectively, reported in previous studies as necessary to reduce the bacterial burden in lungs by 2 log (for colistin treatment) and 3 log (for tigecycline treatment) in experimental models of pneumonia caused by *A. baumannii*, *E. coli*, and *Klebsiella pneumoniae* (23, 24). Final dosing of total imipenem was adjusted to achieve a $\%T_{MIC}$ of at least 40% of the dosing interval (25).

Experimental murine model of peritoneal sepsis. A previously characterized murine model of peritoneal sepsis caused by *A. baumannii* was used (14). Briefly, animals were inoculated i.p. with 0.5 ml of the 100% minimal lethal dose (MLD_{100}) of the Ab9 or Ab186 strain, mixed 1:1 with 10% porcine mucin (Sigma, Spain). The MLD_{100}, the 50% lethal dose (LD_{50}), and the LD_0, the maximum inoculum resulting in no mortality, were determined by inoculating groups of 6 mice i.p. with decreasing concentrations of *A. baumannii*, from 8.78 to 2.3 log CFU/ml for the Ab9 strain and from 8.59 to 2.4 log CFU/ml for the Ab186 strain, and monitoring the survival of the mice for 7 days. LD_{50} values for the strains used in challenge studies were determined using the Probit method. LPC therapy was administered as a pretreatment 1 h before bacterial inoculation, and antimicrobial therapy was initiated 4 h after bacterial inoculation. Groups of 15 mice were randomly ascribed to the following groups: (i) controls (without treatment), (ii) LPC administered once i.p. at 25 mg/kg 1 h before bacterial inoculation, (iii) colistin administered i.p. at 20 mg/kg/8 h for 72 h, (iv) tigecycline administered s.c. at 5 mg/kg/12 h for 72 h, (v) imipenem administered i.m. at 30 mg/kg/4 h for 24 h, (vi) the combination of LPC with colistin, (vii) the combination of LPC with tigecycline, and (viii) the combination of LPC with imipenem. The antimicrobial dosages were chosen after obtaining the PK/PD data.

Mortality was recorded over 24 h (for imipenem treatment groups) or 72 h (for colistin and tigecycline treatment groups). After the death or euthanization of the mice at the end of the experimental period, aseptic thoracotomies were performed, and blood samples for qualitative blood cultures were obtained by cardiac puncture. Samples were inoculated into sterile tubes with 1 ml of LB and incubated for 24 h at 37°C, and then 100 μl was plated onto sheep blood agar. The results of the blood cultures are expressed as positive (when ≥1 CFU was present on the plate) or negative. The spleen and lungs were aseptically removed and homogenized (Stomacher 80; Tekmar Co.) in 2 ml of sterile 0.9% NaCl solution. Tenfold dilutions of the homogenized spleen and lungs were plated onto sheep blood agar for quantitative cultures (to determine the $\log_{10}$ CFU per gram of spleen or lung). If no growth was observed after plating the whole residue of the homogenized tissue, a logarithm value corresponding to the limit of detection of the method (1 CFU) was assigned. The limit of detection could then be determined as follows: CFU/g = 1 CFU/[homogenized volume (ml) + weight (g)].

Experimental murine model of pneumonia. A previously described experimental murine pneumonia model (8) was used to evaluate the efficacy of LPC as monotherapy and in combination with antimicrobial agents against the Ab186 strain. Briefly, the mice were anesthetized by an i.p. injection of 5% (wt/vol) sodium thiopental (Braun Medical, Barcelona, Spain). The mice were suspended vertically, and the trachea of each was then cannulated with a blunt-tipped metal needle. The feel of the needle tip against the tracheal cartilage confirmed the intratracheal location. A microliter syringe (Hamilton Co., Reno, NV) was used for inoculation of 50 μl of a bacterial suspension (≈9 log CFU/ml) which had been grown for 24 h in LB broth at 37°C and mixed at a 1:1 ratio with a 0.9% NaCl solution containing 10% (wt/vol) porcine mucin. The mice remained in a vertical position for 3 min and then in a 30° position until they awakened. Treatment groups were similar to those for the experimental model of peritoneal sepsis. After death or sacrifice of the mice at the end of the experimental period, aseptic thoracotomies were performed, and blood samples for qualitative blood culture were obtained by cardiac puncture (data are reported as numbers [%] of positive cultures). The lungs were aseptically removed and homogenized as described above for quantitative culture (data are reported in $\log_{10}$ CFU per gram of lung).

Cytokine assay. Blood samples were collected from the periorbital plexuses of 30 anesthetized mice infected with the Ab9 and Ab186 strains at the MLD_{100}, as previously described (14). Serum levels of tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) were determined for mice at 0, 2, 4, 8, and 12 h postinfection by using an enzyme-linked immunosorbent assay (ELISA) (eBioscience).

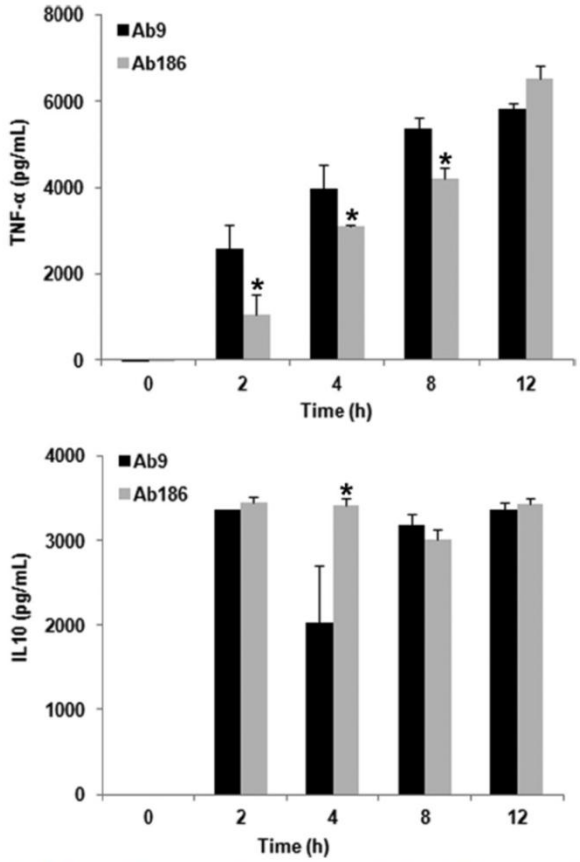

FIG 1 Bacterial effects on cytokine production. Levels of TNF-α (top) and IL-10 (bottom) in serum were determined from 0 to 12 h for mice inoculated with the Ab9 and Ab186 strains. Representative results are shown, and the data are presented as means ± SEM. *, $P < 0.05$ for comparison of Ab9 and Ab186.

Statistical analysis. Group data are presented as means ± standard errors of the means (SEM). For cytokine determination, the Student *t* test was used to determine differences between means. Differences in bacterial spleen and lung concentrations (mean ± SEM log CFU per gram of tissue) were assessed by analysis of variance (ANOVA) and the *post hoc* Bonferroni test. Differences in mortality (%) and blood sterility (%) between groups were compared by use of the χ^2 test. *P* values of <0.05 were considered significant. The SPSS (version 17.0; SPSS Inc.) statistical package was used.

RESULTS

Antimicrobial susceptibilities. The MICs of colistin, tigecycline, imipenem, and LPC for the Ab9 strain were ≤0.5, ≤0.25, ≤0.5, and 8,000 mg/liter, respectively. The MICs of colistin, tigecycline, imipenem, and LPC for the Ab186 strain were ≤0.5, 4, 16, and >8,000 mg/liter, respectively.

Bacterial effects on cytokine production. The effects of *A. baumannii* Ab9 and Ab186 on the serum TNF-α and IL-10 levels were examined from 0 to 12 h after induction of murine peritoneal sepsis (Fig. 1). The Ab9 strain induced more TNF-α release than the Ab186 strain from 0 to 8 h (Fig. 1, top panel), whereas the release of IL-10 at 4 h was significantly smaller with strain Ab9 than with strain Ab186 ($P < 0.05$) (Fig. 1, bottom panel).

Pharmacokinetic and pharmacodynamic parameters. The pharmacokinetic and pharmacodynamic data for total and free colistin, total tigecycline, and total imipenem are shown in Table 1.

MLD_{100}, LD_{50}, and LD_0 of *A. baumannii*. To determine the MLD_{100}, LD_{50}, and LD_0 of strains Ab9 and Ab186, the murine peritoneal sepsis model was used. Mortality was dependent on the concentration of bacteria in the inoculum (data not shown). The MLD_{100}, LD_{50}, and LD_0 of strain Ab9 were 5.9, 4.05, and 2.3 $\log_{10}$ CFU/ml, respectively, and the MLD_{100}, LD_{50}, and LD_0 of strain Ab186 were 5, 3.8, and 2.4 $\log_{10}$ CFU/ml, respectively. The LD_{50} of ATCC 17978 is 2.85 $\log_{10}$ CFU/ml (26).

Efficacies of LPC combination treatments in murine experimental model of peritoneal sepsis. The efficacies of LPC in combination with antimicrobial treatments, expressed as survival, bacterial concentrations in spleens and lungs, and percentages of sterile blood cultures, are shown in Tables 2 to 4.

(i) Survival. Tables 2 and 3, as well as Fig. S1 in the supplemental material, show that all treatments, alone or in combination, increased mouse survival compared with that of the control group for strains ATCC 17978 and Ab9 ($P < 0.05$). Pretreatment with LPC plus treatment with colistin or tigecycline increased mouse survival with strain ATCC 17978 compared to that obtained with pretreatment with LPC ($P < 0.05$) (Table 2). Pretreatment with LPC or treatment with LPC plus colistin, tigecycline, or imipenem increased mouse survival with strain Ab186 compared to that of the control group ($P < 0.05$).

(ii) Bacterial clearance from the spleen and lungs. Tables 2 to 4 show that LPC in combination with colistin decreased spleen

TABLE 1 Pharmacokinetic and pharmacodynamic parameters for single doses of colistin methanesulfonate, tigecycline, and imipenem[a]

Antimicrobial (dose [mg/kg], route of administration)	Drug form	C_{max} (mg/liter)	$t_{1/2}$ (h)	AUC_{0-24} (mg · h/liter)	AUC_{0-24}/MIC[b]		T_{MIC} (h, %)[b]	
					Ab9	Ab186	Ab9	Ab186
CMS (20, i.p.)	CMS	14.24	1.12	43.24	172.97	172.97	ND	ND
	*t*CST	2.87	1.1	14.41	57.63	57.63	ND	ND
	*f*CST	0.97	1.01	4.7	18.81	18.81	ND	ND
TGC (5, s.c.)	*t*TGC	1.34	2.04	13.75	55	3.44	ND	ND
IMP (30, i.m.)	*t*IMP	26.66	0.36	ND	ND	ND	1.54, 38.5	0.09, 2.25

[a] CMS, colistin methanesulfonate; TGC, tigecycline; IMP, imipenem; CST, colistin; *t*CST, total colistin; *f*CST, free colistin; *t*TGC, total tigecycline; *t*IMP, total imipenem; C_{max}, maximum concentration of antimicrobial agent in serum; $t_{1/2}$, elimination half-life; AUC_{0-24}, area under the concentration-time curve from time zero to 24 h; T_{MIC}, time that the drug concentration remains above the MIC; ND, not determined; i.p., intraperitoneal; s.c., subcutaneous; i.m., intramuscular.

[b] For Ab9, the CST, TGC, and IMP MICs were 0.25, 0.25, and 0.5 mg/liter, respectively. For Ab186, the CST, TGC, and IMP MICs were 0.25, 4, and 16 mg/liter, respectively.

TABLE 2 Therapeutic effect of LPC in combination with colistin, tigecycline, or imipenem in murine peritoneal sepsis model with *A. baumannii* ATCC 17978

Treatment[a]	*n*	Log_{10} CFU/g (mean ± SEM)		% sterile blood cultures	Mouse survival (%)[b]
		Spleen	Lungs		
CTL	13	9.72 ± 0.09	8.74 ± 0.18	0	0**
LPC	15	5.81 ± 0.72[c]	6.37 ± 0.62[c]	60[c]	40[c]**
CST	15	0[c,d]	0[c,d]	0[c,d]	100[c,d]**
LPC+CST	15	0[c,d]	0[c,d]	0[c,d]	100[c,d]**
TGC	15	3.00 ± 0.57[c,d]	4.60 ± 0.60[c,d]	93.33[c,d]	93.33[c,d]**
LPC+TGC	15	0.35 ± 0.35[c,d,e]	2.66 ± 0.58[c,d,e]	100[c,d]**	100[c,d]**
IMP	15	0[c,d]	0[c,d]	0[c,d]	100[d]*
LPC+IMP	15	0[c,d]	0[c,d]	0[c,d]	100[e]*

[a] CTL, control (no treatment); LPC, lysophosphatidylcholine; CST, colistin; TGC, tigecycline; IMP, imipenem.
[b] *, mouse mortality was recorded over 24 h; **, mouse mortality was recorded over 72 h.
[c] $P < 0.05$ compared to the controls.
[d] $P < 0.05$ compared to the LPC group.
[e] $P < 0.05$ compared to the TGC group.

TABLE 4 Therapeutic effect of LPC in combination with colistin, tigecycline, or imipenem in murine peritoneal sepsis model with *A. baumannii* Ab186

Treatment[a]	*n*	Log_{10} CFU/g (mean ± SEM)		% sterile blood cultures	Mouse survival (%)[b]
		Spleen	Lungs		
CTL	13	9.79 ± 0.06	9.63 ± 0.13	0	0*
LPC	15	7.73 ± 0.49	7.45 ± 0.48	46.67[c]	46.67[c]**
CST	15	5.48 ± 0.09[c]	6.45 ± 0.84[c]	53.33[c]	53.33[c]**
LPC+CST	15	3.71 ± 1.09[c,d]	3.90 ± 1.12[c,d]	66.67[c]	66.67[c]**
TGC	15	9.60 ± 0.07	9.59 ± 0.13	0[d]	0[d]**
LPC+TGC	15	6.81 ± 0.98[c,e]	6.47 ± 1.06[c,e]	33.33[c,e]	33.33[c,e]**
IMP	15	9.33 ± 0.10[c]	9.60 ± 0.06[d]	0[d]	0[d]*
LPC+IMP	15	6.61 ± 0.65[c,d]	7.36 ± 0.75[c,e]	33.33[c,e]	33.33[c,e]*

[a] CTL, control (no treatment); LPC, lysophosphatidylcholine; CST, colistin; TGC, tigecycline; IMP, imipenem.
[b] *, mouse mortality was recorded over 24 h; **, mouse mortality was recorded over 72 h.
[c] $P < 0.05$ compared to the controls.
[d] $P < 0.05$ compared to the LPC group.
[e] $P < 0.05$ compared to the TGC or IMP group.

and lung concentrations of ATCC 17978, Ab9, and Ab186 by 9.72 and 8.74 log_{10} CFU/g ($P < 0.05$; ATCC 17978), respectively, 9.4 and 6.91 log CFU/g ($P < 0.05$; Ab9), respectively, and 6.08 and 5.73 log CFU/g ($P < 0.05$; Ab186), respectively, compared with the levels for the control groups. In contrast, monotherapy with colistin cleared ATCC 17978, Ab9, and Ab186 from the spleen and lungs by 9.72 and 8.74 log_{10} CFU/g ($P < 0.05$; ATCC 17978), respectively, 6.14 and 6.23 log CFU/g ($P < 0.05$; Ab9), respectively, and 4.31 and 3.18 log CFU/g ($P < 0.05$; Ab186), respectively, compared with the levels for the control groups. LPC in combination with colistin decreased spleen and lung concentrations of ATCC 17978, Ab9, and Ab186 by 5.81 and 6.37 log_{10} CFU/g ($P < 0.05$; ATCC 17978), respectively, 6.87 and 4.08 log CFU/g ($P < 0.05$; Ab9), respectively, and 4.02 and 3.55 log CFU/g ($P < 0.05$; Ab186), respectively, compared with the levels for the LPC pretreatment group.

LPC in combination with tigecycline decreased spleen and lung concentrations of ATCC 17978, Ab9, and Ab186 by 9.37 and 6.08 log_{10} CFU/g ($P < 0.05$; ATCC 17978), respectively, 9.23 and 9.01 log_{10} CFU/g ($P < 0.05$; Ab9), respectively, and 2.98 and 3.16 log_{10} CFU/g ($P < 0.05$; Ab186), respectively, compared with the levels for the control groups. In contrast, tigecycline reduced the spleen and lung concentrations of ATCC 17978, Ab9, and Ab186 by 6.72 and 4.14 log_{10} CFU/g ($P < 0.05$; ATCC 17978), respectively, 8.57 and 8.66 log CFU/g ($P < 0.05$; Ab9), respectively, and 0.19 and 0.04 log_{10} CFU/g (Ab186), respectively, compared with the levels for the control groups. LPC in combination with tigecycline decreased spleen and lung concentrations of ATCC 17978, Ab9, and Ab186 by 5.46 and 3.71 log_{10} CFU/g ($P < 0.05$; ATCC 17978), respectively, 6.7 and 6.18 log CFU/g ($P < 0.05$; Ab9), respectively, and 0.92 and 0.98 log CFU/g (Ab186), respectively, compared with the levels for the LPC pretreatment group.

Finally, LPC in combination with imipenem decreased spleen and lung concentrations of ATCC 17978, Ab9, and Ab186 by 9.72 and 8.74 log_{10} CFU/g ($P < 0.05$; ATCC 17978), respectively, 8.41 and 8.74 log_{10} CFU/g ($P < 0.05$; Ab9), respectively, and 3.18 and 2.27 log_{10} CFU/g ($P < 0.05$; Ab186), respectively, compared with the levels for the control groups. In contrast, imipenem reduced spleen and lung concentrations of ATCC 17978, Ab9, and Ab186 by 9.72 and 8.74 log_{10} CFU/g ($P < 0.05$; ATCC 17978), respectively, 8.32 and 8.21 log_{10} CFU/g ($P < 0.05$; Ab9), respectively, and 0.46 and 0.03 log CFU/g (Ab186), respectively, compared with the levels for the controls. LPC in combination with imipenem decreased spleen and lung concentrations of ATCC 17978, Ab9, and Ab186 by 5.81 and 6.37 log_{10} CFU/g ($P < 0.05$; ATCC 17978), respectively, 5.88 and 5.91 log CFU/g ($P < 0.05$; Ab9), respectively, and 1.12 and 0.09 log CFU/g (Ab186), respectively, compared with the levels for the LPC pretreatment group.

Interestingly, the calculation of mean values for bacterial burdens of dead and live mice separately within the same treatment group for the ATCC 17978, Ab9, and Ab186 strains showed differences (see Tables S1 to S3 in the supplemental material). For the group receiving LPC in combination with colistin against strain Ab186, dead mice ($n = 5$) had higher bacterial burdens (9.3 ± 0.31 log CFU/g in the spleen and 9.66 ± 0.14 log CFU/g in the lungs) than those of live mice ($n = 10$) (0.92 ± 0.38 log_{10} CFU/g in the spleen and 1.02 ± 0.43 log_{10} CFU/g in the lungs) (see Table S3). Similarly, for the group receiving LPC in combination with

TABLE 3 Therapeutic effect of LPC in combination with colistin, tigecycline, or imipenem in murine peritoneal sepsis model with *A. baumannii* Ab9

Treatment[a]	*n*	Log_{10} CFU/g (mean ± SEM)		% sterile blood cultures	Mouse survival (%)[b]
		Spleen	Lungs		
CTL	10	9.55 ± 0.09	9.85 ± 0.72	0	0**
LPC	15	7.02 ± 0.64[c]	7.02 ± 0.67[c]	53.33[c]	53.33[c]**
CST	15	3.41 ± 0.54[c,d]	3.62 ± 0.57[c,d]	53.33[d]	86.67[d]**
LPC+CST	15	0.15 ± 0.15[c,d,e]	2.94 ± 0.31[c,d,e]	100[c,e]	100[c,e]**
TGC	15	0.98 ± 0.58[c,d]	1.19 ± 0.54[c,d]	93.33[c]	93.33[c]**
LPC+TGC	15	0.32 ± 0.24[c,d]	0.84 ± 0.38[c,d]	100[c]	100[c]**
IMP	13	1.23 ± 0.38[c,d]	1.64 ± 0.33[c,d]	100[c]	100[c]*
LPC+IMP	13	1.14 ± 0.39[c,d]	1.11 ± 0.31[c,d]	100[c]	100[c]*

[a] CTL, control (no treatment); LPC, lysophosphatidylcholine; CST, colistin; TGC, tigecycline; IMP, imipenem.
[b] *, mouse mortality was recorded over 24 h; **, mouse mortality was recorded over 72 h.
[c] $P < 0.05$ compared to the controls.
[d] $P < 0.05$ compared to the LPC group.
[e] $P < 0.05$ compared to the CST group.

TABLE 5 Therapeutic effect of LPC in combination with colistin, tigecycline, or imipenem in murine pneumonia model with *A. baumannii* Ab186

Treatment[a]	*n*	Log_{10} CFU/g lung (mean ± SEM)	% sterile blood cultures	Mouse survival (%)[b]
CTL	8	10.93 ± 0.17	0	0*
LPC	8	6.17 ± 0.98[c]	50[c]	50[c]**
CST	15	5.74 ± 1.05[c]	26.67[d]	60[c]**
LPC+CST	15	3.91 ± 0.88[c,e]	73.33[c,e]	73.33[c]**
TGC	14	8.00 ± 0.94	50[c]	50[c]**
LPC+TGC	15	5.11 ± 0.94[c,e]	66.67[c]	66.67[c]**
IMP	12	6.85 ± 0.44[c]	25[c,d]	100[c]*
LPC+IMP	15	4.42 ± 0.75[c,e]	26.67[c]	100[c]*

[a] CTL, control (no treatment); LPC, lysophosphatidylcholine; CST, colistin; TGC, tigecycline; IMP, imipenem.
[b] *, mouse mortality was recorded over 24 h; **, mouse mortality was recorded over 72 h.
[c] $P < 0.05$ compared to the controls.
[d] $P < 0.05$ compared to the LPC group.
[e] $P < 0.05$ compared to the CST, TGC, or IMP group.

tigecycline against strain Ab186, dead mice ($n = 10$) had higher bacterial burdens (9.33 ± 0.08 log_{10} CFU/g in the spleen and 9.22 ± 0.97 log_{10} CFU/g in the lungs) than those of live mice ($n = 5$) (1.78 ± 0.75 log_{10} CFU/g in the spleen and 0.97 ± 0.61 log_{10} CFU/g in the lungs) (see Table S2). For the group receiving LPC in combination with imipenem against strain Ab186, dead mice ($n = 10$) had higher bacterial burdens (8.28 ± 0.18 log_{10} CFU/g in the spleen and 9.34 ± 0.1 log_{10} CFU/g in the lungs) than those of live mice ($n = 5$) (3.26 ± 0.14 log_{10} CFU/g in the spleen and 3.39 ± 0.15 log_{10} CFU/g in the lungs) (see Table S3). Similar results were observed with ATCC 17978 and Ab9 (see Tables S1 and S2).

(iii) Bacterial clearance from blood. For ATCC 17978, LPC in combination with colistin, imipenem, or tigecycline and colistin, imipenem, and tigecycline monotherapies increased the percentages of sterile blood cultures compared with those for the LPC and control groups ($P < 0.05$) (Table 2). For strain Ab9, LPC in combination with colistin, tigecycline, or imipenem increased the percentages of sterile blood cultures compared with those for the colistin, tigecycline, imipenem, LPC, and control groups ($P < 0.05$) (Table 3). For strain Ab186, smaller numbers of mice had detectable bacteria in the blood for LPC in combination with colistin than for the colistin, LPC, and control groups (66.67% versus 53.33%, 46.67%, and 0% sterile blood cultures, respectively). In contrast, LPC in combination with tigecycline or imipenem presented a trend toward fewer sterile blood cultures than those obtained with LPC monotherapy (33.33% for both combinations versus 46.67% for monotherapy) (Table 4).

Efficacies of LPC combination treatments in murine experimental model of pneumonia. The efficacies of LPC in combination with antimicrobial treatments against the MDR Ab186 strain, expressed as survival, bacterial concentrations in the spleen and lungs, and percentages of sterile blood cultures, are shown in Table 5.

(i) Survival. Figure S2 in the supplemental material shows that all treatments, alone or in combination, increased mouse survival of infection with the Ab186 strain compared to that for the control group ($P < 0.05$).

(ii) Bacterial clearance from lungs. Table 5 shows that LPC in combination with colistin and colistin monotherapy decreased the lung concentration of the Ab186 strain by 7.02 and 5.19 log_{10} CFU/g ($P < 0.05$), respectively, compared with the level for the control group. LPC in combination with imipenem and imipenem monotherapy decreased the lung concentration of the Ab186 strain by 6.51 and 4.08 log_{10} CFU/g ($P < 0.05$), respectively, compared with the level for the control group. LPC in combination with tigecycline and tigecycline monotherapy decreased the lung concentration of the Ab186 strain by 5.82 log_{10} CFU/g ($P < 0.05$) and 2.93 log_{10} CFU/g, respectively, compared with the level for the control group. Finally, LPC in combination with colistin, tigecycline, or imipenem decreased the lung concentration of strain Ab186 by 2.26, 1.06, and 1.75 log_{10} CFU/g ($P < 0.05$), respectively, compared with the level for the LPC pretreatment group.

Interestingly, the calculation of mean values for bacterial burdens of dead and live mice separately within the same treatment group for strain Ab186 showed results similar to those for the murine peritoneal sepsis model with Ab186 (see Table S4 in the supplemental material).

(iii) Bacterial clearance from blood. LPC in combination with colistin increased the percentage of sterile blood cultures compared with those for colistin and LPC monotherapies and the control group ($P < 0.05$). LPC in combination with tigecycline presented a trend toward fewer sterile blood cultures than those with tigecycline and LPC monotherapies (33.33% versus 50% and 50%, respectively). In contrast, LPC in combination with imipenem did not increase the sterility of blood cultures compared to that with imipenem monotherapy (Table 5).

DISCUSSION

Previous studies from our group demonstrated that preemptive LPC therapy protected mice from *A. baumannii* infections, reducing bacterial organ loads and bacteremia and increasing mouse survival to 40% (14), similar to the results obtained with both strains used in the present study. In addition, we showed that surviving mice had lower bacterial burdens in tissues than those in dead mice, which explains the observed improvement in survival. Based on this positive therapeutic effect of LPC, we hypothesized that it may be an adjuvant to antimicrobial therapy for patients at risk of severe *A. baumannii* infection (14). Thus, murine experimental models of peritoneal sepsis and pneumonia were performed to evaluate the efficacy of LPC in combination with colistin, tigecycline, or imipenem.

Nowadays, the high prevalence of MDR *A. baumannii* reduces treatment options. Colistin and tigecycline are among the last treatments available worldwide, due to their low resistance rates (27, 28). Imipenem remains the gold standard treatment for infections with susceptible strains, and it is widely used in the clinical setting (29). In the present study, monotherapy with colistin against susceptible and MDR *A. baumannii* strains significantly reduced bacterial spleen and lung concentrations and bacteremia and increased mouse survival.

Interestingly, in the experimental model of peritoneal sepsis, pretreatment with LPC in combination with colistin treatment reduced the bacterial loads in tissues and the proportion of bacteremia compared to those for colistin treatment alone. Accordingly, the LPC-colistin combination also increased mouse survival. Both *A. baumannii* strains are susceptible to colistin; however, the spleen and lung bacterial loads were 2 log higher for the MDR strain than for the susceptible strain after treatment with

colistin or pretreatment with LPC plus treatment with colistin. This difference in bacterial load was not due to different pharmacodynamics between the strains, because the MIC of Ab9 and Ab186 was 0.5 μg/ml, which is equal to the MIC for both strains before inoculation into mice. We suggest that the difference in bacterial loads may be due to the difference in immune responses caused by both strains. Indeed, Fig. 1 shows that the susceptible strain induced more TNF-α release than that of the MDR strain. These data are in agreement with those previously reported from our group showing that a susceptible *A. baumannii* strain induced more TNF-α and IL-6 release than that by MDR and pan-drug-resistant *A. baumannii* clinical isolates (30, 31). In line with these data, macrophages deficient in Toll-like receptor 4 (TLR4) or neutrophil depletion resulted in impaired bacterial killing ability against *A. baumannii*, possibly due at least to the alteration of production of proinflammatory cytokines (TNF-α, IL-17, gamma interferon [IFN-γ], and IL-1β) (32–34). In an *in vivo* study, Qiu et al. associated the susceptibility of mice to *A. baumannii* infection with reduced local proinflammatory cytokine responses, including TNF-α responses, and with a delay in the early influx of neutrophils into the lung (35). In contrast, inhibition of lipopolysaccharide (LPS) by an LpxC inhibitor suppressed *A. baumannii* LPS-mediated activation of TLR4 and consequently reduced inflammation *in vivo* (36).

With respect to the usefulness of the combination of LPC with tigecycline or imipenem, we observed a trend toward a decrease in bacterial tissue concentrations compared to those with antimicrobial monotherapies, but without statistical significance. In the case of the experimental model of peritoneal sepsis caused by the susceptible Ab9 strain, the high efficacy of imipenem or tigecycline alone (bacterial concentrations in tissues were ≈1 to 1.5 $\log_{10}$ CFU/g) precluded the observation of a larger reduction with the combined treatment. In the case of the experimental models of peritoneal sepsis and pneumonia caused by the MDR strain, as expected, no therapeutic effect was observed with tigecycline or imipenem treatment due to resistance to both antimicrobials. However, in mice treated with LPC in combination with tigecycline or imipenem, bacterial loads were significantly reduced compared to those for the controls and the tigecycline- or imipenem-treated group. Although LPC plus imipenem or tigecycline reduced the bacterial concentrations in tissues ≈1 to 2 $\log_{10}$ CFU/g compared to those with LPC monotherapy, these differences were not significant, as the results were not different in terms of bacteremia and survival. These data show that LPC has a moderate impact when there is no antibiotic efficacy because of high drug resistance.

The LPC prophylactic treatment model has some limitations regarding the animal model system and the LPC treatment regimens. We believe that the next issues to be addressed are as follows: (i) to determine the bacterial burdens in tissues and bacteremia only for live and moribund mice to avoid any effect of death for both control and treated groups in order to confirm the therapeutic efficacy of LPC in combination with antimicrobial agents observed in the present study; (ii) to determine whether multiple doses of LPC given as a treatment in combination with antimicrobial agents can improve the effect of LPC; and (iii) to use BALB/c mice as another animal model, since BALB/c mice are Th2 biased, while C57BL/C6 mice are Th1 biased (37).

It is important that other immunomodulation applications have been performed, such as application of granulocyte colony-stimulating factor (G-CSF) as an adjuvant with antibiotics and application of corticosteroids as an adjunct treatment for pneumonia (38, 39). Positive results have been seen in animals for both applications, but when mixed with clinical results, G-CSF application does not mimic the results seen with the animal model (38).

In summary, the present study suggests that LPC pretreatment in combination with colistin, tigecycline, or imipenem treatment improves the *in vivo* antibacterial activity in cases of experimental peritoneal sepsis and pneumonia caused by *A. baumannii*.

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Chapter II. Article II: supplementary material

Supplemental data

Table S1. Therapeutic effect of LPC in combination with colistin, tigecycline or imipenem in murine peritoneal sepsis model of *A. baumannii* ATCC 17978 strain.

Treatment	n	Log_{10} CFU/g spleen (mean ± SEM)		Log_{10} CFU/g lung (mean ± SEM)	
		Dead (n)	Alive (n)	Dead (n)	Alive (n)
CTL	13	9.72 ± 0.09 (13)	--	8.74 ± 0.18 (13)	--
LPC	15	7.56 ± 0.54 (9)	3.18 ± 0.81 (6)	7.96 ± 0.41 (9)	3.97 ± 0.6 (6)
CST	15	--	0 (15)	--	0 (15)
LPC+CST	15	--	0 (15)	--	0 (15)
TGC	15	8.29 (1)	2.63 ± 0.45 (14)	10.81 (1)	4.15 ± 0.44 (14)
LPC+TGC	15	--	0.35 ± 0.35 (15)	--	2.66 ± 0.58 (15)
IMP	15	--	0 (15)	--	0 (15)
LPC+IMP	13	--	0 (15)	--	0 (15)

CTL, control (no treatment); LPC, lysophosphatidylcholine; CST, colistin; TGC, tigecycline; IMP, imipenem.

Table S2. Therapeutic effect of LPC in combination with colistin, tigecycline or imipenem in murine peritoneal sepsis model of *A. baumannii* Ab9 strain.

Treatment	n	Log_{10} CFU/g spleen (mean ± SEM)		Log_{10} CFU/g lung (mean ± SEM)	
		Dead (n)	Alive (n)	Dead (n)	Alive (n)
CTL	9	9.55 ± 0.09 (9)	--	9.85 ± 0.72 (9)	--
LPC	15	9.28 ± 0.23 (7)	5.05 ± 0.56 (8)	9.34 ± 0.13 (7)	5.00 ± 0.65 (8)
CST	15	7.53 ± 1.93 (2)	2.66 ± 0.32 (13)	7.82 ± 1.89 (2)	2.75 ± 0.29 (13)
LPC+CST	15	--	0.15 ± 0.15 (15)	--	2.94 ± 0.31 (15)
TGC	15	8.79 (1)	0.43 ± 0.19 (14)	7.9 (1)	0.71 ± 0.28 (14)
LPC+TGC	15	--	0.32 ± 0.25 (15)	--	0.84 ± 0.38 (15)
IMP	13	--	1.23 ± 0.39 (13)	--	1.64 ± 0.33 (13)
LPC+IMP	13	--	1.14 ± 0.39 (13)	--	1.11 ± 0.31 (13)

CTL, control (no treatment); LPC, lysophosphatidylcholine; CST, colistin; TGC, tigecycline; IMP, imipenem.

Table S3. Therapeutic effect of LPC in combination with colistin, tigecycline or imipenem in murine peritoneal sepsis model of *A. baumannii* Ab186 strain.

Treatment	n	Log_{10} CFU/g spleen (mean ± SEM)		Log_{10} CFU/g lung (mean ± SEM)	
		Dead (n)	Alive (n)	Dead (n)	Alive (n)
CTL	13	9.79 ± 0.06 (13)	--	9.63 ± 0.13 (13)	--
LPC	15	9.88 ± 0.07 (8)	5.24 ± 0.71 (7)	9.94 ± 0.12 (8)	4.95 ± 0.75 (7)
CST	15	8.89 ± 0.46 (7)	2.49 ± 0.43 (8)	9.68 ± 0.18 (7)	3.63 ± 0.46 (7)
LPC+CST	15	9.3 ± 0.31 (5)	0.92 ± 0.38 (10)	9.66 ± 0.14 (5)	1.02 ± 0.43 (10)
TGC	15	9.6 ± 0.07 (15)	--	9.59 0 ± 0.13 (15)	--
LPC+TGC	15	9.33 ± 0.08 (10)	1.78 ± 0.75 (5)	9.22 ± 0.1 (10)	0.97 ± 0.61 (5)
IMP	15	9.33 ± 0.1 (15)	--	9.6 0 ± 0.06 (15)	--
LPC+IMP	15	8.28 ± 0.18 (10)	3.26 ± 0.14 (5)	9.34 ± 0.1 (10)	3.39 ± 0.15 (5)

CTL, control (no treatment); LPC, lysophosphatidylcholine; CST, colistin; TGC, tigecycline; IMP, imipenem.

Table S4. Therapeutic effect of LPC in combination with colistin, tigecycline or imipenem in murine pneumonia model of *A. baumannii* Ab186 strain.

Treatment	n	Log_{10} CFU/g lung (mean ± SEM)	
		Dead (n)	Alive (n)
CTL	8	10.93 ± 0.17 (8)	--
LPC	8	10.32 (1)	3.30 ± 0.50 (7)
CST	15	10.10 ± 0.47 (6)	2.82 ± 0.67 (9)
LPC+CST	15	8.58 ± 0.68 (4)	2.21 ± 0.59 (11)
TGC	14	11.14 ± 0.04 (7)	4.91 ± 0.78 (7)
LPC+TGC	15	10.77 ± 0.12 (5)	2.29 ± 0.84 (10)
IMP	12	--	6.85± 0.44 (12)
LPC+IMP	15	--	4.42 ± 0.92 (15)

CTL, control (no treatment); LPC, lysophosphatidylcholine; CST, colistin; TGC, tigecycline; IMP, imipenem.

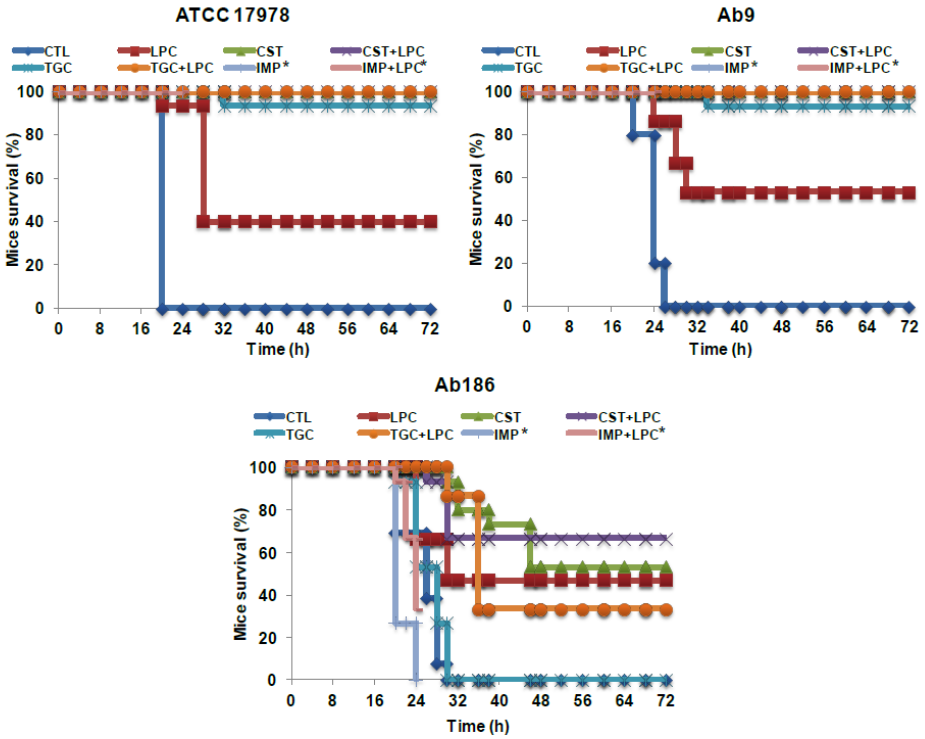

Figure S1. Mice survival after treatment with LPC in combination with colistin, tigecycline or imipenem in peritoneal sepsis model by ATCC 17978, Ab9 or Ab186. CTL, control (no treatment); LPC, lysophosphatidylcholine; CST, colistin; TGC, tigecycline; IMP, imipenem.
*: mice mortality was recorded over 24 h in imipenem w/o LPC.

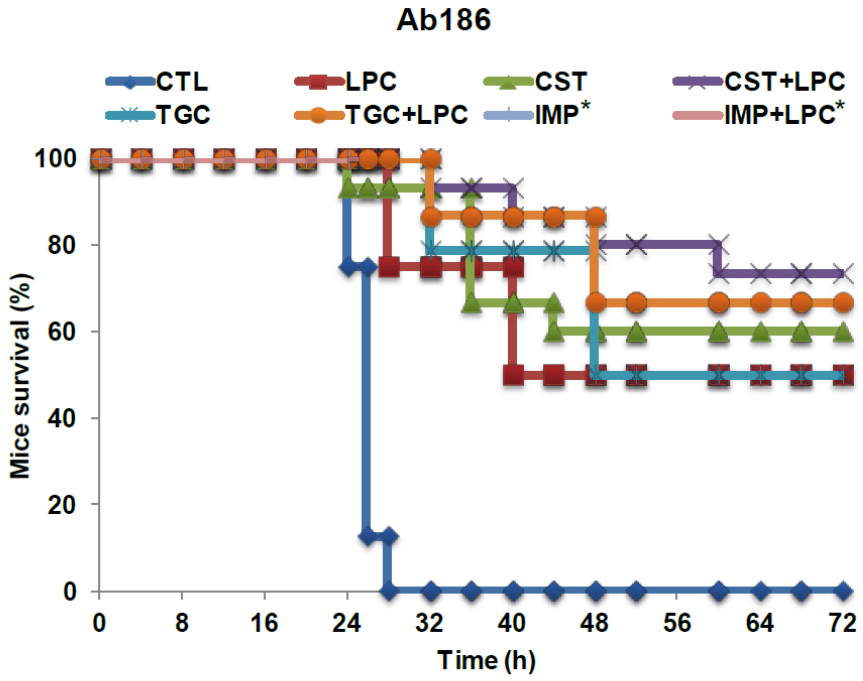

Figure S2. Mice survival after treatment with LPC in combination with colistin, tigecycline or imipenem in pneumonia model by Ab186. CTL, control (no treatment); LPC, lysophosphatidylcholine; CST, colistin; TGC, tigecycline; IMP, imipenem.
*: mice mortality was recorded over 24 h in imipenem w/o LPC.

Chapter II. Article III

2.2. Article III. Efficacy of lysophosphatidylcholine alone and in combination with antimicrobial agents in experimental murine peritoneal sepsis and pneumonia models by *Pseudomonas aeruginosa*

Since the significant increase of the antimicrobial resistance of *P. aeruginosa* and the lack of efficient treatments for the infections that it causes, the searching of new alternatives is mandatory. The stimulation of the immune system response to reduce the progression of the infection, in combination with antimicrobial treatment, would be an alternative. In our group we have previously demonstrated that LPC, an immunomodulator involved in immune cells recruitment and activation, in monotherapy and combined with antibiotics confers protection against susceptible and MDR *A. baumannii*. Consequently, we aimed to evaluate the therapeutic efficacy of LPC and LPC in combination with ceftazidime or imipenem in murine experimental models of peritoneal sepsis and pneumonia by clinical susceptible and MDR *P. aeruginosa*.

P. aeruginosa strain susceptible to imipenem and ceftazidime, and *P. aeruginosa* MDR strain resistant to imipenem and ceftazidime were used. Pharmacokinetics and pharmacodynamics parameters of ceftazidime, and minimal lethal doses (MLD) of both strains were determined. In murine experimental models of peritoneal sepsis and pneumonia, mice were pretreated with one dose of LPC at 75 mg/kg, infected with MLD of susceptible or MDR strain, and combined or not with imipenem (30 mg/kg/4 h) during 24 h or ceftazidime (100 mg/kg/12 h) during 72 h, and compared with ceftazidime and imipenem monotherapies, respectively, and infected control groups without treatments. Bacterial load in spleen and lungs, bacteremia and mice survival were analyzed. Furthermore, the levels of pro- and anti-inflammatory cytokines, TNF-α and IL-10, respectively, were determined by ELISA in murine experimental models of peritoneal sepsis and pneumonia by *P. aeruginosa* MDR strain.

With respect to the MDR strain, LPC in combination with imipenem reduces spleen and lung bacterial loads and bacteremia, and increases mice survival in both experimental models. LPC in combination with ceftazidime reduces spleen and lung bacterial loads and bacteremia in both models, and significantly decreases mortality rates in the pneumonia model; however, it does not improve mice survival in the peritoneal sepsis model.

Regarding the cytokines release, antimicrobial monotherapies increased and decreased significantly the TNF-α and IL-10, respectively, compared with the controls

at 8 h post-inoculation. Meanwhile, LPC-antimicrobial combinations decreased and increased significantly the TNF-α and IL-10 levels, respectively, in comparison with the controls and with monotherapies.

Chapter II. Article III

Title: Efficacy of lysophosphatidylcholine alone and in combination with antimicrobial agents in experimental murine peritoneal sepsis and pneumonia models by *Pseudomonas aeruginosa*.

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Short running title: LPC therapeutic effect alone & in combination therapy.

Keywords: Lysophosphatidylcholine, combined antimicrobial treatment, peritoneal sepsis model, pneumonia model, *Pseudomonas aeruginosa*.

Chapter II. Article III

ABSTRACT

Lysophosphatidylcholine (LPC), an immunomodulator involved in immune cells recruitment and activation, in monotherapy and in combination therapy with antibiotics confers protection against multidrug-resistant (MDR) *Acinetobacter baumannii*. In this report, we showed that LPC in combination with ceftazidime or imipenem, in murine peritoneal sepsis and pneumonia models by clinical MDR *Pseudomonas aeruginosa* isolates, tends to improve the antibacterial activity of ceftazidime and imipenem by reducing bacterial tissue and blood concentrations and increasing mice survival.

TEXT

Pseudomonas aeruginosa is one of the most important bacteria associated with nosocomial infections such as pneumonia, skin and soft tissue infections, urinary tract infection, ocular infection, bacteremia, septicemia and endocarditis (1). During the last decade this pathogen has become increasingly resistant to most antimicrobials, including imipenem and ceftazidime (2,3). The limitation of antimicrobial alternatives in the optimal treatment of severe infections by multidrug-resistant (MDR) *P. aeruginosa* prompts the urgent search for alternative therapeutic options (3,4). The immune system stimulation by lysophosphatidylcholine (LPC) (5-7) is one of the promising approaches which has demonstrated efficacy against *Acinetobacter baumannii* in monotherapy or in combination with colistin, tigecycline or imipenem (8,9). In this study, we aimed to evaluate the efficacy of LPC alone and in combination with imipenem or ceftazidime in murine experimental models of peritoneal sepsis and pneumonia by susceptible (Pa39) and MDR (Pa238) *P. aeruginosa* strains. Expanded details of materials and methods and statistical analysis are given in the supplemental materials.

The MICs of imipenem, ceftazidime and LPC for Pa39 were 1, 1, and >8000 mg/L, respectively, and for Pa238 were 32, 64, and >8000 mg/L, respectively. The pharmacokinetic and pharmacodynamics data are shown in Table 1.

The efficacy of LPC alone and in combination with imipenem or ceftazidime in murine (female C57BL/6) peritoneal sepsis model after inoculation with 3.85 $\log_{10}$ CFU/mL (100% lethal dose [LD_{100}] of Pa39 or 6.7 $\log_{10}$ CFU/mL (LD_{100}) of Pa238 is shown in Table 2. All treatments, except LPC alone, increased survival compared with the controls for Pa39 ($p<0.05$). For Pa238, only LPC+imipenem increased survival compared with the controls ($p<0.05$). Regarding the spleen and lungs bacterial loads, imipenem or ceftazidime monotherapies decreased those of Pa39 and Pa238 in ≈7.5 and 0.5 $\log_{10}$ CFU/g ($p<0.05$) or in ≈7.5 and 1.3 $\log_{10}$ CFU/g ($p<0.05$), respectively compared with the controls. LPC+imipenem decreased those of Pa39 or Pa238 in ≈8

and 2 log_{10} CFU/g ($p<0.05$) or in ≈8 and 2 log_{10} CFU/g ($p<0.05$), respectively, compared with the controls or LPC monotherapy. LPC+ceftazidime decreased bacterial loads of Pa39 or Pa238 by ≈8 and 4.7-5 log_{10} CFU/g ($p<0.05$) or by ≈8 and 4.5 log_{10} CFU/g ($p<0.05$) respectively, compared with the controls or LPC monotherapy. With respect to the bacteremia by Pa39, imipenem and ceftazidime monotherapies reduced it to 0%; for Pa238, only LPC+imipenem and LPC+ceftazdime reduced it to 93.33% and 53.33%, respectively, compared with the controls ($p<0.05$).

Additionally, the efficacy of LPC alone and in combination with imipenem or ceftazidime was tested in the murine pneumonia model after inoculation of 10 log_{10} CFU/mL (LD_{100}) of each strain (Table 3). For both strains, treatment with LPC+imipenem or LPC+ceftazidime reduced mortality to 7%-64% compared with the controls. Regarding the bacterial lungs load, imipenem or ceftazidime decreased Pa39 and Pa238 by 4.87 and 2.43 log_{10} CFU/g ($p<0.05$) or 4.48 ($p<0.05$) and 1.26 log_{10} CFU/g, respectively, compared with the controls. LPC+imipenem decreased bacterial lungs load of Pa39 and Pa238 by 5.91 and 4.89 log_{10} CFU/g ($p<0.05$), or 4.88 ($p<0.05$) and 2.79 log_{10} CFU/g, compared with the controls or LPC monotherapy. LPC+ceftazidime decreased bacterial lungs load of Pa39 and Pa238 by 5.02 ($p<0.05$) and 3.63 log_{10} CFU/g, or in 3.99 ($p<0.05$) and 0.49 log_{10} CFU/g, respectively, compared with the controls or LPC monotherapy. With respect to bacteremia, LPC+imipenem and LPC+ceftazidime reduced it by both strains to ≈50%-93% compared with the controls, and to ≈20%-40% compared with the antimicrobial monotherapies ($p<0.05$).

The effects of different treatments on cytokine production, in peritoneal sepsis and pneumonia models were evaluated. Imipenem and ceftazidime monotherapies increased and decreased significantly the TNF-α and IL-10 serum levels, respectively, in comparison with the controls at 8 h post-inoculation. Meanwhile, both combinations of LPC+antimicrobials decreased and increased significantly the TNF-α and IL-10 levels, respectively, in comparison with the controls

and with monotherapies (Fig. 1). These results are in accordance with the previously reported immunomodulatory effects of LPC (7,8).

This study shows that imipenem and ceftazidime monotherapies in both models, by the susceptible Pa39 and the MDR Pa238 strains, reduced differently the bacterial tissues concentrations and bacteremia, and increased survival, in concordance with their antimicrobial activities.

In the pneumonia model by both strains, treatment with LPC+imipenem or LPC+ceftazidime did not reduce the bacterial lungs load, bacteremia, nor mortality, when compared with antimicrobial monotherapies. In the case of the MDR strain, as expected, no therapeutic effect was observed with imipenem or ceftazidime due to resistance to both antimicrobials; however, the treatment with LPC+imipenem or LPC+ceftazidime, reduced bacterial loads and bacteremia by ≈1.35-2.35 $\log_{10}$ CFU/g and 50%, respectively, compared with antimicrobial monotherapies; and survival increased slightly. Similar data has been observed with LPC+imipenem or LPC+tigecycline in the *A. baumannii* resistant to imipenem and tigecycline pneumonia model (9).

In the peritoneal sepsis model by the MDR Pa238 strain LPC+ceftazidime did not reduce survival at 72 h, even if the bacterial burden in tissue was lower than in controls and ceftazidime monotherapy; the analysis of survival at 24 h showed a mortality of 27% (4 out of 15 mice), mortality similar to the 20% at 24 h with LPC+imipenem. This data suggests that LPC, administered in one dose previous to the inoculation, only reduced early mice mortality.

Interestingly, comparing the effect of the pro-inflammatory cytokine TNF-α, the combination of LPC+imipenem or LPC+ceftazidime reduced their levels by 83- or 82-folds, respectively, in the peritoneal sepsis model by MDR strain. In contrast, these reductions were lower in the pneumonia model: 7- or 28-folds with LPC+imipenem or LPC+ceftazidime, respectively. These differences in the anti-inflammatory effect of LPC in both models could be the cause of the different results in terms of mice survival.

The present study has the limitation that LPC was administered in only one dose, before the bacterial inoculation; thus, its immunomodulatory effect is probably in a reduced period. However, these results warrant performing additional studies to determine whether multiple doses of LPC in combination with antimicrobial agents, may improve the effect of LPC in combination with antimicrobials against Gram-negative bacilli infections.

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Table 1. Pharmacokinetic and pharmacodynamic parameters of single doses of imipenem and ceftazidime

Antimicrobial (dose, route of administaration	Drug	C_{max} (mg/L)	$T_{1/2}$ (h)	AUC_{0-24h} (mg.h/L)	*T>MIC* (h, %)		References
					Pa39	Pa238	
IMP (30 mg/kg, im)	*t*IMP	26.66	0.36	11.23	1.5, 37.5%[a]	0, 0%[b]	(9)
CFZ (100 mg/kg, ip)	*f*CFZ	107.14	1.08	126.83	6, 50%[a]	1, 8.33%[b]	This study

IMP, imipenem; *t*IMP: total imipenem, CFZ: ceftazidime; *f*CFZ: free ceftazidime; C_{max}, maximum concentration of antimicrobial agent in serum; $T_{1/2}$, elimination half-time; AUC_{0-24h}, area under the concentration-time curve from time 0 to 24h; T>MIC, the time that drug concentration remains above the MIC; im: intramuscular ; ip: intraperitoneal.

[a]IMP and CFZ MICs: 1 mg/L.

[b]IMP and CFZ MICs: 32 and 64 mg/L, respectively.

Table 2. Therapeutic effect of LPC in combination with imipenem or ceftazidime in murine peritoneal sepsis model of *P. aeruginosa*.

Strain	Treatment	N	Log_{10} CFU/g of spleen (mean ± SEM)	Log_{10} CFU/g of lungs (mean ± SEM)	Positive blood cultures (%)	Mortality (% [N])	Time to death[#] (Md ± SEM, h)
Pa39	CTL	8	8.04 ± 0.06	7.78 ± 0.10	100	100 (8)**	21.38 ± 0.38
	LPC	8	$8.57 \pm 0.09_{a}$	7.89 ± 0.07	100	100 (8)**	22.25 ± 0.16
	IMP	14	$0.54 \pm 0.29_{a,b}$	$0.14 \pm 0.14_{a,b}$	$0_{a,b}$	$0\ (0)^{*}_{a,b}$	>24
	LPC + IMP	15	$0.17 \pm 0.17_{a,b}$	$0.14 \pm 0.14_{a,b}$	$0_{a,b}$	$0\ (0)^{*}_{a,b}$	>24
	CFZ	14	$0.49 \pm 0.49_{a,b}$	$0.49 \pm 0.49_{a,b}$	$0_{a,b}$	$7\ (1)^{**}_{a,b}$	47 ± 0.00
	LPC + CFZ	15	$0_{a,b}$	$0_{a,b}$	$0_{a,b}$	$0\ (0)^{*}_{a,b}$	>72
Pa238	CTL	8	8.91 ± 0.29	8.42 ± 0.35	100	100 (8)**	21.5 ± 0.00
	LPC	8	8.68 ± 0.09	8.06 ± 0.21	100	100 (8)**	23.14 ± 0.96
	IMP	14	8.40 ± 0.11	7.78 ± 0.10	100	100 (14)**	18.82 ± 1.12
	LPC + IMP	15	6.74 ± 0.74	6.33 ± 0.7	93	$20\ (3)^{*}_{a,b,c}$	20 ± 2.31
	CFZ	14	$7.67 \pm 0{,}21_{a,b}$	$6.90 \pm 0.24_{a,b}$	100	100 (14)**	34.18 ± 1.94
	LPC + CFZ	15	$4.22 \pm 0.58_{a,b,c}$	$3.34 \pm 0.59_{a,b,c}$	$53_{a,b,c}$	100 (15)**	47.47 ± 4.94

CTL, controls (no treatment); LPC, lysophosphatidylcholine; IMP, imipenem; CFZ, ceftazidime.
*: mice mortality was recorded over 24 h, **: mice mortality was recorded over 72 h, [#]: time to mice death
[a]: compared to the controls, $P < 0.05$;; [b]: compared to LPC, $P < 0.05$, [c]: compared to the IMP or CFZ, $P < 0.05$.

Table 3. Therapeutic effect of LPC in combination with imipenem or ceftazidime in murine pneumonia model of *P. aeruginosa*.

	Treatment	N	Log_{10} CFU/g of lungs (mean ± SEM)	Positive blood culture (%)	Mortality (% [N])	Time to death[#] (mean ± SEM, h)
Pa39	CTL	8	9.44 ± 0.19	100	100 (8)**	21 ± 0
	LPC	13	8.41 ± 0.72	92	92 (12)**	20.67 ± 2.58
	IMP	15	$4.57 \pm 0.56_{a,b}$	$47_{a,b}$	$53\ (8)^{*}_{a,b}$	9 ± 0.65
	LPC + IMP	15	$3.53 \pm 0.60_{a,b}$	$7_{a,b,c}$	$47\ (7)^{*}_{a,b}$	10.29 ± 1.19
	CFZ	14	$4.96 \pm 0.93_{a,b}$	64	64 (9)**	22.22 ± 3.47
	LPC + CFZ	14	$4.42 \pm 0.93_{a,b}$	$43_{a,b}$	64 (9)**	16.22 ± 0.22
Pa238	CTL	8	8.38 ± 0.73	100	37 (3)*	23.33 ± 0.67
	LPC	8	8.74 ± 0.83	100	37 (3)*	22.67 ± 1.33
	IMP	14	7.30 ± 0.68	$57_{a,b}$	14 (2)*	21.5 ± 1.5
	LPC + IMP	14	$5.95 \pm 0.83_{a}$	$21_{a,b}$	7 (1)*	23 ± 0
Pa238	CTL	8	10.84 ± 0.07	100	100 (8)**	29.75 ± 3.1
	LPC	15	7.7 ± 1.11	73	$60\ (9)^{**}_{a}$	25 ± 2.12
	CFZ	15	9.58 ± 0.53	93	87 (13)**	30.62 ± 3.99
	LPC + CFZ	15	$7.21 \pm 0.79_{a}$	$53_{a,c}$	$60\ (9)^{**}_{a}$	31.33 ± 2.85

CTL, controls (no treatment); LPC, lysophosphatidylcholine; IMP, imipenem; CFZ, ceftazidime.
*: mice mortality was recorded over 24 h, **: mice mortality was recorded over 72 h, [#]: time to mice death.
[a]: compared to the controls, $P < 0.05$; [b]: compared to the LPC, $P < 0.05$; [c]: compared to the IMP or CFZ, $P < 0.05$.

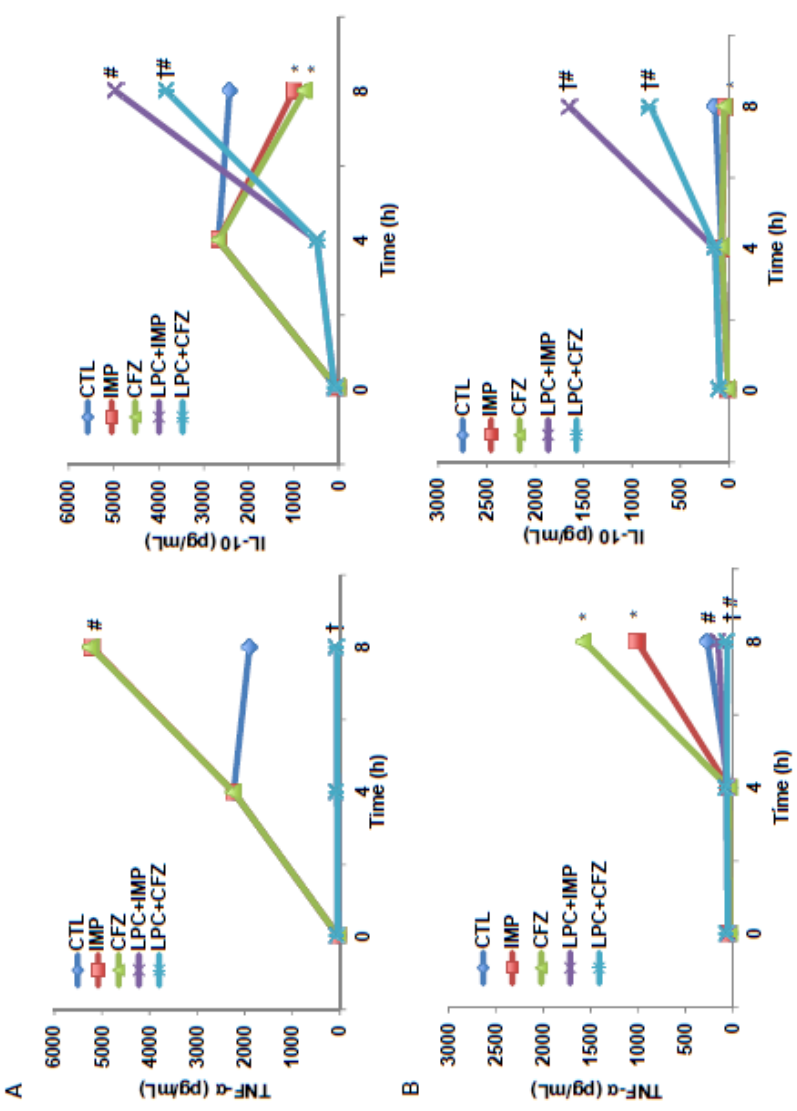

Figure 1. Cytokine production after 8 h of MDR *P. aeruginosa*-induced peritoneal sepsis (A) and pneumonia (B). Levels of TNF-α and IL-10 in serum were determined from 0 to 8 h for mice inoculated with the Pa238 strain and treated or not with imipenem, ceftazidime, LPC-imipenem combination or LPC-ceftazidime combination. Representative results are shown, and the data are presented as means. CTL, controls (no treatment); LPC, lysophosphatidylcholine; IMP, imipenem; CFZ, ceftazidime. * and †: compared to CTL, $P < 0.05$; #: compared to IMP or CFZ. $P < 0.05$.

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Supplementary data

MATERIALS AND METHODS.

Bacterial strains. 1) *P. aeruginosa* (Pa39) clinical strain susceptible to ceftazidime and imipenem isolated from blood culture, and 2) MDR *P. aeruginosa* (Pa238) clinical strain, resistant to ceftazidime, imipenem, ciprofloxacin and tobramycin, isolated from blood culture were used. Both strains were from the REIPI-GEIH 2008 collection (1).

Antimicrobial agents and reagents. For the *in vitro* assays, antimicrobials were used as standard laboratory powders: ceftazidime and imipenem (Sigma, Spain). For the *in vivo* experiments, clinical formulations of antimicrobials were used: ceftazidime (Normon, Spain) and imipenem (Merk Sharp & Dohme, Spain). The anesthetic was 5% (w/v) sodium thiopental administered intraperitoneally (i.p.) (B. Braun Medical S.A., Spain).

***In vitro* susceptibility testing.** Minimal inhibitory concentrations (MICs) were determined by broth microdilution assay according to standard CLSI recommendations (2), as previously described (3). *Escherichia coli* ATCC 25922 was used as a control strain.

Animals. Immunocompetent C57BL/6 female mice, weighting 18 to 20 g (Production and Experimentation Animal Center, University of Seville, Seville, Spain) were used. Animals were housed in regulation cages and given free access to food and water. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (4). The protocol was approved by the

Committee of Ethics for Animal Experiments of the University Hospital of Virgen del Rocío of Seville (2014PI/014).

Antimicrobial pharmacokinetics and pharmacodynamics parameters. Serum LPC and imipenem levels were previously determined by our research group (5,6). Serum ceftazidime levels were determined in groups of 30 healthy mice following single doses of 100 mg/kg i.p. ceftazidime.

In sets of three animals at 0, 5, 10, 15, 30, 60, 90, 120, 240, 480 and 1440 min after antimicrobial administration, blood samples were obtained from anesthetized mice from periorbital plexus. Concentrations of ceftazidime were measured using a HPLC-tandem mass spectrometry (LC-MS/MS) (7). The C_{max} in serum, $AUC_{0-\infty}$, $t_{1/2}$, and $T_{>MIC}$ ratios were obtained by a computer-assisted method (8). Final dosing of total or free ceftazidime in the *in vivo* experiments was adjusted to achieve a $T_{>MIC}$ at least ≈ 40%-50% of the dosing interval (9).

Experimental murine model of peritoneal sepsis. The previously characterized murine peritoneal sepsis model by *A. baumannii* was used (5). Briefly, animals were inoculated i.p. with 0.5 ml of the minimal lethal dose 100 (MLD_{100}) of Pa39 or Pa238 strains, mixed 1:1 with 10% porcine mucin (Sigma, Spain). The MLD_{100}, lethal dose 50 (LD_{50}) and lethal dose 0 (LD_0) were determined by inoculating groups of 6 mice i.p. with decreasing concentrations of *P. aeruginosa* from 7.6 to 3.85 Log CFU/ml for Pa39 strain, and from 7.8 to 4 Log CFU/ml for Pa238 strain, and monitoring the survival of the mice for 7 days; these values were determined using the Probit method. LPC therapy was administered as a pretreatment 1 h before bacterial inoculation, and antimicrobial therapy was initiated 4 h after bacterial inoculation. Groups of 15 mice were randomly

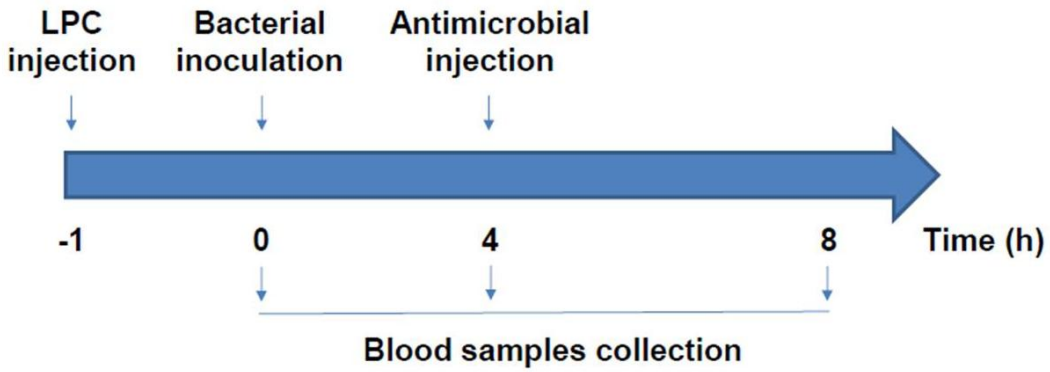
ascribed to the following groups: 1) controls (without treatment), 2) LPC administered once at 75 mg/kg i.p. 1 h before bacterial inoculation, 3) ceftazidime administered i.p. at 100 mg/kg/12 h for 72 h, 4) imipenem administered i.m. at 30 mg/kg/4 h for 24 h, and 5 and 6) the combinations of LPC at 75 mg/kg and ceftazidime at 100 mg/kg/12 h, and imipenem at 30 mg/kg/4 h, respectively. The antimicrobial dosages were chosen after obtaining the PK/PD data.

Mortality was recorded over 24 h (for imipenem treatment groups) or 72 h (for ceftazidime treatment group). After the death or the putting down of the mice at the end of the experimental period, aseptic thoracotomies were performed, and blood samples were obtained by cardiac puncture for qualitative blood cultures. Samples were inoculated in sterile tubes with 1 ml of Luria Bertani (LB) broth and incubated for 24 h at 37 °C, and then 100 µl were plated onto sheep blood agar. The results of the blood cultures are expressed as positive (when $\geq$ 1 CFU was present in the plate) or negative. The spleen and lungs were aseptically removed and homogenized (Stomacher 80; Tekmar Co., USA) in 2 ml of sterile NaCl 0.9 % solution. Ten-fold dilutions of the homogenized spleen and lungs were plated onto sheep blood agar for quantitative cultures (Log_{10} CFU/g of spleen or lung).

Experimental murine model of pneumonia. A previously described experimental murine pneumonia model (10) was used to evaluate the efficacy of LPC in monotherapy and in combination with antimicrobial agents against Pa39 and Pa238 strains. Briefly, the mice were anesthetized by an i.p. injection of 5% (wt/vol) sodium thiopental. They were suspended vertically, and the trachea of each was then cannulated with a blunt-tipped metal needle. The fell of the needle tip against the tracheal cartilage confirmed the intratracheal location. A microliter syringe (Hamilton Co., Reno, NV) was used for

inoculation of 50 µl of a MLD_{100} bacterial suspension (≈10 log CFU/ml). This MLD_{100} was obtained after 24 h of bacterial growth in LB broth at 37°C. The mice remained in a vertical position for 3 min and then in a 30° position until awake. Treatment groups were similar to the experimental model of peritoneal sepsis. After death or putting down of the mice at the end of the experimental period, aseptic thoracotomies were performed, and blood samples for qualitative blood culture were obtained by cardiac puncture (data are reported as numbers [%] of positive cultures). The lungs were aseptically removed and homogenized as described above for quantitative culture (data are reported in Log_{10} CFU/g of lung).

Cytokine assay. Blood samples were collected from the periorbital plexuses of 60 anesthetized mice infected with the Pa238 at the MLD100 in the peritoneal sepsis and pneumonia models and treated or not with imipenem, ceftazidimie, LPC-imipenem combination or LPC-ceftazidime combination, as previously described (6). Serum levels of tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) were determined in mice at 0, 4, and 8 h postinfection by using an enzyme-linked immunosorbent assay (ELISA) (eBioscience) following this protocol scheme:



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Statistical analysis. Differences in bacterial spleen and lungs concentrations (mean ± standard error of the mean (SEM) log CFU/g of tissue) were assessed by analysis of variance (ANOVA) and post-hoc Dunnett test. Differences in mortality (%) and blood sterility (%) between groups were compared by χ^2 test after normalization determination by Kolmogorov-Smirnov test. A p-value of <0.05 was considered significant. The SPSS (version 17.0) statistical package was used (SPSS Inc).

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VI. DISCUSSION

Discussion

Nowadays, the population worldwide is facing a very important problem due to the resistance of most bacteria against the antibiotics currently available to treat infections, which has risen rapidly and steadily in the last decades. Consequently, there is an urgent need for new antibiotics or compounds active against Gram-negative microorganisms. Adjuvant therapies include: antibiotic combinations, synergy between antibiotics and non-antibiotics molecules, molecules that alter the physiology of antibiotic-insensitive cells and inhibition of resistance.

Therefore, our work in this doctoral thesis is focused on the prevention and treatment of GNB infections by OmpA inhibitor peptides, and by the stimulation of the immune system. We discuss below the results obtained in this doctoral thesis.

1. Chapter I:

Impact of OmpA inhibition on Gram-negative bacilli virulence and antimicrobial resistance

As formerly explained, therapies combining antimicrobial agents and peptides are among the promising new strategies to treat drug resistant bacterial infections (308). So, as we previously reported that *ompA*-deficient *A. baumannii* is less adherent to biotic and abiotic surfaces (86) and less virulent in murine experimental model of peritoneal sepsis (309) and that recombinant OmpA shows high affinity to fibronectin, an extracellular matrix protein (85), we developed a library of 26 cyclic hexapeptides to computationally screen for OmpA inhibition.

Six peptides with high affinity *in silico*, and one control peptide (SXV4) were synthesized in order to validate the *in silico* screening (**Table S3 and Figure S1, Art. I**). To confirm the activity of the peptides, assays to test their activity blocking the interaction between bacteria (*A. baumannii*, *P. aeruginosa* and *E. coli*) and host cells were performed.

1.1. Therapeutic efficacy of AOA-2 as monotherapy against Gram-negative bacilli

After the initial screening of OmpA inhibitors which consisted in the evaluation of the bactericidal activity, the toxicity in human lung epithelial cells (A549) (**Table S4, Art. I**) and bacterial adherence to host cells (**Figure S2, Art. I**), we selected one compound, AOA-2, for further studies due to its ability reducing bacterial adherence, comparing with the other peptides. It showed a reduction in the adherence of *A. baumannii* to A549 cells by more than 60 % *in vitro* using 0.25 mg/mL of the compound (**Figure 1 C and Figure 1D, Art.I**). Due to highly conservation of OmpA among most of the GNB, the same assay using *P. aeruginosa* and *E. coli* was performed observing a significant reduction in adherence as observed previously (**Figure 1C, Art. I**). As it is known that GNB are able to achieve attachment and invasion of host cells by binding to host-cell fibronectin mediated by OMPs, as OmpA (85), we also tested bacterial interaction with immobilized fibronectin to strengthen adherence results. As expected, bacterial attachment to fibronectin was significantly reduced with AOA-2 treatment (**Figure 1E, Art. I**). However, the percentage of bacterial attachment was slightly different than in the adherence assay, suggesting that AOA-2 may block other factors involved in the attachment of bacteria.

In order to prove that AOA-2 blocks OmpA, we reconstituted OmpA from *E. coli* into planar lipid bilayers to form ion channels as stated by Saint *et al* (307). We verified that the addition of AOA-2 inhibits the conductance of OmpA channels in a concentration-dependent manner, which means that OmpA channels were closed or blocked due to the addition of the peptide to the electrolytic media (**Figure A4, Art. I**). We performed the same experiment with the control peptide SXV1 and its addition showed no difference in the conductance of the channels, even at the highest concentration.

As previously mentioned, the aim of developing these compounds was to inhibit the adherence between bacteria and eukaryotic cells. Therefore, two important features are necessary for these compounds, which are: not showing antibacterial activity by itself and no cytotoxic activity. Accordingly, we determined by time-kill curves that AOA-2 does not present bacteriostatic or bactericidal activities (**Figure S4, Art. I**). Moreover, by MTT assay, none of the peptides showed cytotoxic activities at the concentrations tested (**Table S4, Art. I**).

In addition, it is known that OmpA plays a role in biofilm formation (88), so we tried AOA-2 to inhibit this property. As a result, AOA-2 also diminishes the biofilm formation in *A. baumannii*, *P. aeruginosa* and *E. coli* clinical isolates (**Table S5, Art. I**). In other study, it has been demonstrated the antibiofilm activity of the broad spectrum peptide 1018, which inhibits biofilm formation and also eradicates preformed biofilms formed by MDR GNB (266).

Another property of OmpA is that it is involved in cell death induction after its translocation into the host' nucleus and in mice mortality (82), therefore, *in vitro* and *in vivo* activity of AOA-2 against GNB virulence was studied. A549 cell death was tested in the presence of 0.25 or 0.5 mg/mL of AOA-2 (**Figure 1F, Art. I**). For *A. baumannii* and *P. aeruginosa* AOA-2 significantly prevented cell death dependent on bacterial adherence (81, 107) however, there was an exception with *E. coli* strain ATCC 25922. The fact that AOA-2 does not prevent cell death by *E. coli* infection may be ascribable to the presence of other highly virulent factors circumventing the loss of OmpA, such as toxins (310). To perform the *in vivo* virulence studies in mice, first, it is necessary to test toxicity of AOA-2. Results showed that there was no toxicity at a dose of $\leq$ 40 mg/kg of AOA-2 (**Table S6, Art. I**). Consequently, mice were infected i.p. with MLD of *A. baumannii* ATCC 17978, *P. aeruginosa* Pa01 and *E. coli* ATCC 25922 strains and treated with 10 mg/kg AOA-2 2h after infection. AOA-2 in monotherapy significantly reduced the bacterial spleen and lung load, for the three strains, compared to the untreated groups (**Figure 1G, Art. I**). Furthermore, positive blood cultures and mouse mortality were also significantly decreased compared to the controls (**Figure 1H, Art. I**). Other works has found peptides which shown therapeutic efficacy *in vivo*, such as the peptide Api88, that rescued mice from *E. coli* septicemia infection after three injection of the peptide (311). However, there are some others with good results *in vitro* but not *in vivo*, as Cecropin A-melitin that showed short-term efficacy in PDR *A. baumannii sepsis* model (245). In order to prove the bioavailability of AOA-2 when administered at 10 mg/kg i.p. to mice, we studied the pharmacokinetics in healthy mice, which indicated that AOA-2 showed good activity (**Figure S5, Art. I**).

In summary, we have developed an hexacyclic peptide which is able to inhibit the virulence of *A. baumannii*, *P. aeruginosa* and *E. coli* strains both *in vitro* and *in vivo* by blocking the binding of bacteria OmpA to host-cell fibronectin.

1.1.Therapeutic efficacy of AOA-2 as combined therapy

After the establishment that AOA-2 is very effective against GNB, we thought that the efficacy of this compound could be improved by co-administering it with antimicrobial agents, as the combination of molecules to block resistance and enhance activity of antibiotics represents a highly promising strategy that can contribute to this need. In fact, combined therapy with antimicrobial agents and peptides is among the encouraging new strategies to treat drug resistant bacterial infections (308).

The contribution of OmpA in the antimicrobial resistance phenotype has been demonstrated. For instance, the disruption of *ompA* gene led to decrease MICs of chloramphenicol, aztreonam, nalidixic acid and polymyxin B in *A. baumannii* (49, 312), as the disruption of *oprF* in *P. aeruginosa* induces susceptibility to novobiocin (90). Therefore we thought it would be interesting to examine the capacity of AOA-2 to potentiate the activity of these antimicrobial agents on *A. baumannii* clinical isolates. In agreement with previous observations (49, 312)**,** MICs data showed that AOA-2 increased susceptibility of ATCC 17978 to chloramphenicol, aztreonam, nalidixic acid and colistin (**Table S7 and Table S8, Art. I**). Moreover, susceptibility to colistin was increased after the addition of AOA-2 to colistin-susceptible and colistin-resistant *A. baumannii* clinical isolates, with the MIC_{50} for colistin decreasing from 32 to 2 μg/mL in the presence of AOA-2 (**Table S8, Art. I**). The same phenomena was stated in *P. aeruginosa* and *E. coli* by Simonetti *et al*. with the peptide IB-367, which reduced MICs values of colistin and imipenem when were combined with the peptide (284).

In order to determine the behavior of the combination between AOA-2 and colistin, time-kill assays using both colistin-susceptible (Col-S) (ATCC 17978) and colistin-resistance (Col-R) (#11) were performed. Time-kill curves showed for Col-S and Col-R strains synergistic and bactericidal activities in the combined treatment of AOA-2 with colistin, improving significantly the decrease of bacterial growth with respect to monotherapy with colistin (**Figure 2A, Art. I**). It is known that the β-barrel assembly machinery (BAM) complex plays a critical role in OMPs biogenesis and Mori *et al*. demonstrated that blocking the subunit BamD in *P. aeruginosa* with a synthetic peptide based on homologous sequences of BamD (FIRL) can potentiate colistin, levofloxacin, erythromycin, vancomycin and rifampicin susceptibilities of this pathogen (313). In our study, we observed a bacterial regrowth occurred 4 h after incubation of AOA-2 with

colistin. This regrowth may be due to the colistin half-life in bacterial culture broth, which is 4 h (314, 315), so it may be degraded at that time. Thus, to maintain the colistin concentration in the following experiments, we added for the second time colistin 4 h post-incubation. This time was chosen because bacterial regrowth at 4h was absent, and the activity and synergy between AOA-2 and colistin was greater (**Figure 2B, Art. I**), mainly for the Col-R strain.

Based on the observed synergy between AOA-2 and colistin against Col-S and Col-R *A. baumannii* strains, we suggested that AOA-2 may regulate the OMPs expression, as reported previously in another study (316). To demonstrate it we decided to analyse the protein profile of OMPs in presence and absence of AOA-2 for *A. baumannii* ATCC 17978 and #11 strains.

SDS-PAGE assays showed that in presence of AOA-2 the expression of a protein, identified as Omp25 by MALDI-TOF-TOF, was increased, and conferred a ≥ 33- and 8-fold reduction of colistin MIC, for ATCC 17978 and #11 strains, respectively (**Figure 2C, Art.I**). To confirm that the Omp25 expression was associated with colistin resistance, *omp25* gene transcription analysis was determined in Col-S and Col-R strains, and showed that *omp25* was less transcripted in Col-R strains (**Figure 2D, Art.I**). RT-PCR studies also confirmed that the expression of *omp25* in Col-R strains was increased when the strains were previously incubated with AOA-2 (**Figure 2D, Art.I**). This data altogether showed that the treatment with AOA-2 become the bacteria more susceptible to colistin treatment, producing a synergy between both compounds and reducing bacterial growth. Due to this synergy, the bacteria may employ additional resistance mechanisms which involve the protein Omp25. We contemplate the possibility that *omp25* gene is overexpressed when bacteria is treated with AOA-2 in order to compensate the inhibition of OmpA, with the aim of allowing nutrients entrance, but at the same time, colistin could be interacting with the membrane through it producing consequently the decrease in bacterial growth.

In order to analyse with more detail the role of this protein on the synergistic activity between AOA-2 and colistin, an *omp25*-deficient mutant (Δ*omp25*) from the ATCC 17978 strain was generated. The deletion of *omp25* in the ATCC 17978 strain increased its colistin MIC with respect to the wild type. To demonstrate that this increment was due exclusively to the loss of *omp25* gene, we complemented the mutant Δ*omp25* to

restore the wild-type phenotype. AOA-2 did not significantly reduce the colistin MIC of the Δ*omp25* strain (2-folds), whereas the colistin MIC was reduced 8.33-fold in the complemented Δ*omp25* mutant (**Figure 2E, Art. I**). In the same line, Mori *et al*. showed that blocking BamD from Col-S *P. aeruginosa* with the peptide FIRL regulates the expression of MexAB-OprM, and the construction of its mutant increases colistin susceptibility (313).

Furthermore, additional experiments were performed to confirm the role of Omp25 in colistin resistance. Time-kill curves showed no synergistic activity with the combined treatment of AOA-2 with colistin for Δ*omp25* strain, meanwhile, as expected for the complemented Δ*omp25* strain, AOA-2 combined treatment with colistin restored almost completely the synergistic activity observed in the wild type strain (**Figure 2F, Art. I**). OMP profile was also performed for the Δ*omp25* and the complemented Δ*omp25* strains to prove the role of AOA-2 on OMPs expression. Results showed that incubation of AOA-2 in Δ*omp25* strain did not reveal the expression of Omp25, however for the complemented Δ*omp25* strain, Omp25 was expressed as in the wild type strain (**Figure 2E, Art.I**).

The next issue was to evaluate the efficacy of AOA-2 in combination with colistin in murine model of peritoneal sepsis. A number of infections caused by MDR strains require the use of colistin, but these bacteria rapidly acquire specific resistance mechanisms against this drug (317). In a clinical trial, treatment with the optimized dose of colistin prevented only 50 % of mortality from patients with *A. baumannii* infections (318). Moreover, in mice, the use of optimal colistin prevents only 33-40 % of mice from mortality (200). The rate of colistin resistance currently varies between 3 and 28 % worldwide (319). In order to confirm the *in vivo* synergistic effects of AOA-2 with colistin against *A. baumannii*, first mice were infected with the MLD of *A. baumannii* ATCC 17978 strain or #11 strains, and after that they were treated with 10 mg/kg AOA-2 in combination with colistin sub-optimal dose 10 mg/kg/d. This combination reduced bacterial load of both strains in spleen and in lungs compared with colistin alone (**Figure 3B, Art. I**). Moreover, mice receiving combined treatment of AOA-2 and colistin showed a greater increase in survival and absence of bacteremia than those receiving colistin alone (**Figure 3C, Art. I**). As previously mentioned, several studies have demonstrated *in vivo* the beneficial effect of adjuvant drugs, such as peptides, in combination with antimicrobials. For instance in murine pneumonia model caused by *P.*

aeruginosa, it has been assessed the combination of the peptide FIRL with colistin or levofloxacin, reducing significantly bacterial burden of the lungs compared with antimicrobial monotherapies (313). Furthermore, in mouse wound infection model caused by *P. aeruginosa* or *E. coli*, the combination of the peptide IB-367 with colistin or imipenem reduced skin bacterial counts in contrast with monotherapies with antimicrobials (284).

Accordingly to this data, the combined treatment of AOA-2 with colistin improves the prognosis of the infection caused by *A. baumannii* in murine model of peritoneal sepsis, suggesting that it might be an appropriate option to treat these infections and that it could help to solve problems related with infections caused by *A. baumannii* Col-S and Col-R. This combination could also prevent the emergence of strains resistant to colistin, due to the reduction on the antimicrobial dose.

2. Chapter II:

Therapeutic efficacy of LPC in combination with antimicrobial agents against Gram-negative bacilli in experimental murine peritoneal sepsis and pneumonia models

As we explained previously, another approach for combating infections by GNB is to enhance host defenses using immunomodulators. It is known that LPC is a natural adjuvant for the immune system, inducing humoral and cellular immune responses (208). It is implicated in immune cell modulation and recruitment (209) such as monocytes (320), phagocytes (321) and T lymphocytes (322). It has been also described in humans that after intracutaneous injection, LPC induces a local inflammation and leukocyte accumulation at the site of injection, demonstrating that it is capable of inducing an inflammatory reaction (323). Moreover, we have previously demonstrated in a study from our group that preemptive LPC therapy protected mice from infections by *A. baumannii*, where tissues bacterial load and bacteremia were reduced, and mice survival was increased to 40 % (210). Therefore, as animal models provide us a way to evaluate new treatments and characterize the host immune response, we have studied *in vivo* the therapeutic efficacy of LPC in combination with clinically used antimicrobials

in murine experimental models of peritoneal sepsis and pneumonia caused by *A. baumannii* or *P. aeruginosa*.

2.1. Therapeutic efficacy of LPC in combination with antimicrobials agents against *A. baumannii in vivo*

At the present time, *A. baumannii* MDR infections are still a serious challenge for clinicians because of its propensity to acquire resistance to a wide spectrum of antimicrobial agents. Its infections are associated with high mortality rates and longer hospital stays. Recently, colistin and tigecycline have emerged as alternative therapeutic options for *A. baumannii* MDR infections (162, 324), and imipenem is the drug of choice for the treatment of serious infections caused by this bacteria (325).

In the second article of the present doctoral thesis, the efficacy of combination treatment of LPC plus colistin, tigecycline or imipenem, in murine experimental models of peritoneal sepsis and pneumonia were evaluated.

We showed that monotherapy with colistin against susceptible and MDR *A. baumannii* strains significantly reduced bacterial spleen and lung concentrations and bacteremia and increased mice survival in murine experimental models of peritoneal sepsis and pneumonia (**Tables 3, and 5, Art. II**). Nevertheless, in both models, the combination of LPC with colistin was able not only to reduce bacterial load in spleen and lungs and the proportion of bacteremia but also to increase mice survival compared to the treatment with colistin alone. Although both *A. baumannii* strains are susceptible to colistin, there was a difference of about 2 Log more in spleen and lung bacterial burden for the MDR strain compared with the susceptible one after treatment with colistin alone and after the combination of LPC with colistin. We discard that this difference could be due to different pharmacodynamics between *A. baumannii* susceptible and MDR strains because the MIC of colistin for both strains after the infection in mice was 0.5 μg/mL, which is the same they had before the inoculation. So we suggested that the difference in bacterial loads may be due to the difference in immune responses caused by both strains. Consequently, we determined the amount of TNF-α and IL-10 on serum after induction of murine peritoneal sepsis caused by *A. baumannii* susceptible and MDR strains. Ab9 susceptible strain induced more TNF-α release than the Ab186 MDR strain,

whereas release of IL-10 was significantly lower with the susceptible strain than with the MDR strain (**Figure 1, Art. II**). These results are in agreement with those previously reported by Smani *et al.* (326, 327) which showed that susceptible *A. baumannii* strain induced more TNF-α and IL-6 release than that by MDR and PDR *A. baumannii* clinical isolates. It is known the importance about the recruitment of cells from the immune system for the host to combat bacterial infections, as neutrophils (328). According to this, it has been demonstrated that macrophages deficient in Toll-like receptor 4 (TLR4) or neutrophil depletion resulted in impaired bacterial killing ability against *A. baumannii*, possibly due at least to the alteration of production of proinflammatory cytokines (TNF-α, IL-17, gamma interferon [IFN-γ], and IL-1β) (328-330). Furthermore, in an *in vivo* study, the susceptibility of mice was associated to *A. baumannii* infection with reduced local proinflammatory cytokine responses, including TNF-α responses, and with a delay in the early influx of neutrophils into the lung (331). In contrast, inhibition of LPS by and LpxC inhibitor suppressed *A. baumannii* LPS-mediated activation of TLR4 and consequently reduced inflammation *in vivo* (236).

With regard to the combination of LPC with tigecycline or imipenem, bacterial burden concentrations tend to decrease compared to antimicrobial monotherapies, but without statistical significance. The fact of lacking statistical significance in the case of the peritoneal sepsis caused by the susceptible strain is that imipenem and tigecycline have high efficacy alone (bacterial concentrations in tissues were ≈ 1 to 1.5 $\log_{10}$ CFU/g) which prevented the observation of a larger reduction with the combined treatment (**Table 3, Art. II**). Otherwise, in the case of the experimental models of peritoneal sepsis and pneumonia caused by the MDR strain no therapeutic effect was observed with imipenem or tigecycline, as expected, due to the resistance to both antimicrobials (**Tables 4 and 5, Art. II**). Moreover, compared to LPC monotherapy, LPC combined with imipenem or tigecycline reduced the bacterial concentrations in tissues about ≈ 1 to 2 $\log_{10}$ CFU/g, but these differences were not significant, as the results were not different in terms of bacteremia and survival. These data show that LPC has a moderate impact when there is no antibiotic efficacy because of high drug resistance.

2.2. Therapeutic efficacy of LPC in combination with antimicrobials agents against *P. aeruginosa in vivo*

Based in the positive therapeutic effect of LPC in monotherapy and in combination therapy with antimicrobial agents against MDR *A. baumannii*, we hypothesized that LPC may also be an adjuvant to the antimicrobial therapy for those patients at risk of severe *P. aeruginosa* infections.

As a consequence, murine experimental peritoneal sepsis and pneumonia models caused by *P. aeruginosa* were performed to evaluate the efficacy of LPC alone and in combination with imipenem or ceftazidime.

Nowadays, the high prevalence of *P. aeruginosa* MDR strains reduces the treatment options, as presents a significant quandary in selecting empiric antimicrobial therapy in severe ill hospitalized patients. Imipenem and ceftazidime are among the treatment option available. Imipenem remains the gold standard treatment for infections by susceptible strains, and it is widely used in the clinical setting (325). Cephalosporins have broad spectrum and low toxicity profile, so they are a popular choice for both targeted and empiric therapy, therefore ceftazidime is used for the treatment of severe GNB infections in hospitals which has a low incidence of MDR strains (332).

In the third article of the present doctoral thesis, the efficacy of combination treatment of LPC plus imipenem or ceftazidime in murine experimental models of peritoneal sepsis and pneumonia were evaluated.

Monotherapy with imipenem or ceftazidime against susceptible *P. aeruginosa* strain in peritoneal sepsis and pneumonia models reduced differently bacterial spleen and lungs concentrations and bacteremia, and increased mice survival (**Table 2 and Table 3, Art. III**).

With respect to the usefulness of the combination of LPC with imipenem or ceftazidime in the experimental model of peritoneal sepsis by MDR strain, we have observed a decrease in the bacterial tissues concentrations and bacteremia compared with antimicrobial monotherapies, and an increase in the mice survival in the case of the treatment with imipenem, but not with ceftazidime in combination with LPC even if the bacterial load and bacteremia were lower (**Table 2, Art. III**). In the same line of this

result, in 2013 Jacqueline *et al.* demonstrated that in murine experimental model of pneumonia by *P. aeruginosa*, the bacterial burden in spleen and lung after treatment with ceftazidime was 2.74 and 4.74 log CFU/mL, and the mortality reached 80 % (333).

Interestingly, in the experimental model of pneumonia by susceptible and MDR *P. aeruginosa*, pretreatment with LPC in combination with imipenem or ceftazidime reduced the bacterial load in tissues and the proportion of bacteremia when compared to imipenem or ceftazidime treatment alone, although without showing statistical significance (**Table 3, Art. III**). Probably, if the number of mice were greater, statistical significance would be achieved. Accordingly, the LPC-imipenem or ceftazidime combination also increased mice survival (**Table 3, Art. III**). As expected for the MDR strain, no therapeutic effect was observed with imipenem or ceftazidime treatment due to resistance to both antimicrobials. However, in mice treated with LPC in combination with imipenem or ceftazidime, bacterial loads and bacteremia were reduced by ≈1.35-2.35 Log CFU/g and 50 %, respectively, compared with the imipenem or ceftazidime treated groups; and the mice survival was increased slightly (**Table 3, Art. III**). Similar data have been observed when LPC was combined with imipenem or tigecycline in experimental model pneumonia by *A. baumannii* resistant to imipenem and tigecycline (334).

It is important to note that ceftazidime in combination with LPC against MDR strain in peritoneal sepsis model did not improve the mice survival than ceftazidime and LPC monotherapy, or control animal (**Table 2, Art. III**). Meanwhile, ceftazidime combined with LPC increased the mice survival in the pneumonia model (**Table 3, Art. III**). In the case of others GNB such as *A. baumannii* ATCC 17978 strain, we found that LPC monotherapy in peritoneal sepsis model only increased 40 % of mice survival vs. the 68% observed in the pneumonia model (334). In the same way, rifampicin combined with colistin did not improve the mice survival in peritoneal sepsis model by carbapenemase-producing *K. pneumoniae* than in pneumonia model (unpublished data). This difference between both experimental models of infections would be due to the severity of peritoneal sepsis model which sepsis was defined as the result of a dysregulated systemic inflammatory response syndrome in the presence of infection, accompanied by major organ failure and death (335).

LPC is a chemotactic factor that stimulates immune cells and regulates the balance between the release of pro- and anti-inflammatory cytokines. Thus, to define the level of bacteriology-associated sepsis in the impact of the modeling system, we determined the release of pro- and anti-inflammatory cytokines in mice serum in both experimental models. Imipenem and ceftazidime monotherapies increased and decreased significantly the TNF-α and IL-10, respectively, compared with the controls at 8 h post-inoculation, for peritoneal sepsis and pneumonia models. Meanwhile, LPC-imipenem and LPC-ceftazidime combinations decreased and increased significantly the TNF-α and IL-10 levels, respectively, in comparison with the controls and with monotherapies (**Figure 1, Art. III**). These results are in accordance with the previous data reported by Smani *et al*. in which LPC pretreatment induced a decrease in the levels of pro-inflammatory cytokines TNF-α and IL-6, whereas the level of the anti-inflammatory cytokine IL-10 was increased (210).

Nevertheless, this LPC prophylactic treatment model has some limitations regarding the treatment regimens of LPC. We believe that the next issue to be addressed is to determine whether multiple doses of LPC, given as treatment in combination with antimicrobial agents, can improve the effect of LPC. It is important to note that other immunomodulatory applications have been performed such as application of granulocyte colony stimulating factor (G-CSF) as an adjuvant with antibiotics, and application of corticosteroids as an adjunct treatment of pneumonia (336, 337). Positive results have been seen in animals with both applications, but when mixed with clinically results G-CSF application does not mimic the results of animal model (336).

To summarize, after the preclinical evaluation of these compounds it is important to note that the use of non-antimicrobial strategies is a promising alternative to prevent and treat infections by MDR GNB restoring the efficacy of existing antibiotics.

We have demonstrated, both *in vitro* and *in vivo*, that by inhibiting GNB OmpA by a synthetic peptide, we are able to block GNB virulence factors, which leads to a protection of the host against infections by these pathogens. In addition, the combination of this peptide with colistin showed a synergistic activity, improving the

prognosis of the infection caused by *A. baumannii* in experimental murine model of peritoneal sepsis.

We have also demonstrated that LPC, which is involved in immune cell recruitment and modulation, has shown promising *in vivo* results in experimental murine models by *A. baumannii* and *P. aeruginosa*. The innate immune response is very important to control bacterial infections and their clinical outcomes. By its modulation, we can improve the body´s ability to eliminate infections via multiple mechanisms.

Employing adjuvants will enable us to use current antimicrobial agents to fight against pathogens that have developed resistance or were never susceptible to certain antimicrobial agents in the first place. Furthermore, the utilization of these adjuvants and antimicrobial agents at the same time would reduce the amount of antimicrobial used for treatments and consequently, the chances of developing resistance.

VII. CONCLUSIONS

Conclusions

1. The inhibition of OmpA by AOA-2 reduces the bacterial adherence of *A. baumannii, P. aeruginosa* and *E. coli* to host cells by reducing their affinity to fibronectin, and the formation of biofilm.
2. The inhibition of OmpA by AOA-2 decreases the cytotoxicity of host cells by *A. baumannii* and *P. aeruginosa* infection.
3. The monotherapy with AOA-2 can protect against the infections by *A. baumannii, P. aeruginosa* and *E. coli* in murine experimental model of peritoneal sepsis by reducing their bacterial load in tissues, the bacteremia and by increasing the mice survival.
4. The inhibition of OmpA by AOA-2 increases the *in vitro* synergistic activity with colistin against colistin susceptible and resistant *A. baumannii*.
5. A possible additional bacterial resistance mechanism to colistin which depends on Omp25 is characterized in *A. baumannii* and possibly involved in the synergistic activity between AOA-2 and colistin.
6. The combined therapy between AOA-2 and colistin improves the prognosis of the infection caused by colistin susceptible and resistant *A. baumannii* in murine experimental model of peritoneal sepsis.
7. AOA-2 may to be suggested as a novel class of antimicrobial agents that has proven to be effective as a monotherapy and in combination with current antimicrobial treatment in experimental model of infection by GNB.
8. The combined therapy between LPC and colistin, tigecycline or imipenem improves the evolution of the infection caused by susceptible and MDR *A. baumannii*, respectively, in experimental murine model of peritoneal sepsis and pneumoniae.
9. The host inflammatory response induced by peritoneal sepsis and caused by *A. baumannii* susceptible strain is different from that caused by the *A. baumannii* MDR strain.
10. The combined therapy between LPC and imipenem or ceftazidime reduces spleen and lung bacterial load and bacteremia against susceptible and MDR *P. aeruginosa* in murine peritoneal sepsis and pneumonia models, and increases mice survival against both strains in murine pneumonia model.
11. Monotherapy with imipenem or ceftazidime, and combined therapy between LPC and imipenem or ceftazidime, respectively, increases and decreases significantly the release of the pro-inflammatory cytokine TNF-α induced by MDR *P.*

aeruginosa in murine peritoneal sepsis and pneumonia compared to that released by infected mice without antimicrobial treatment.

12. Monotherapy with imipenem or ceftazidime, and combined therapy between LPC and imipenem or ceftazidime, respectively, reduces and increases significantly the release of the anti-inflammatory cytokine IL-10 induced by MDR *P. aeruginosa* in murine peritoneal sepsis and pneumonia compared to that released by infected mice without antimicrobial treatment.
13. Combination therapy between LPC and current antimicrobial treatments improves the evolution of the infection caused by GNB in severe infections murine experimental models.

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